Immune responses to BCG immunisation and Mycobacterium tuberculosis infection in non-human primates

Thesis

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Immune responses to BCG immunisation and *Mycobacterium tuberculosis* infection in non-human primates

by

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Abstract:

The evaluation of new vaccines that are urgently needed against tuberculosis (TB) is hampered by the lack of an immune correlate of protection. One of the main measures of immune function utilised in TB vaccine studies is the assessment of interferon (IFN)-γ, as it is known that this cytokine is essential for immunity against infection.

The studies conducted in this thesis uses the non-human primate model of TB to address whether two measurements of IFN-γ (the frequency of IFN-γ secreting cells and the concentration of IFN-γ secreted) can be related to survival and thus provide a correlate of protection. The *Mycobacterium*-specific IFN-γ profiles have been assessed in two primate species (rhesus macaques and cynomolgus macaques) following immunisation with BCG, or aerosol infection with *Mycobacterium tuberculosis*. Additionally, one of the most advanced novel TB vaccines (modified vaccinia virus Ankara expressing antigen 85A; MVA85A) has also been tested in this model.

When human clinical trial data (provided by Oxford University) using BCG and BCG with an MVA85A boost were compared with results generated in similarly vaccinated macaques, it was observed that rhesus macaques have kinetically similar IFN-γ responses, but at different magnitudes. During a vaccine efficacy study, rhesus macaques were shown to be protected from *M. tuberculosis* infection by BCG vaccination. This allowed the opportunity for responses in animals protected against disease to be compared with those which were not protected.

Although results showed that the capability to secrete larger concentrations of PPD-specific IFN-γ early after *M. tuberculosis* infection correlated with longer survival periods, this was not solely due to prior vaccination. Most of the IFN-γ responses during the vaccination periods did not relate to survival after challenge, except peak frequencies of IFN-γ secreting cells after BCG vaccination. Therefore, it was concluded that measuring IFN-γ alone does not provide the correlate of protection that is presently lacking in TB vaccine research.

Declaration:

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All work with non-human primates was carried out by staff in the Biological Investigations Group at the Centre for Emergency Preparedness and Response (CEPR), part of the Health Protection Agency.

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Abbreviations

AdAg85A	Adenovirus expressing antigen 85A
APC	Antigen presenting cell
aa	Amino acids
Ag85A	Antigen 85A
AIDS	Acquired immune deficiency syndrome
ABTS	2,2'-azino-di[3-ethylbenzthiazolin sulphate]
BCG	Bacille Calmette Guérin
BPRC	Biomedical Primate Research Centre
BSA	Bovine serum albumin
BCIP/NBT	5-Bromo-4-Chloro-Indolyl Phosphate/Nitro Blue Tetrazolium
BAL	Broncho-alveolar lavage
CEPR	Centre for Emergency Preparedness and Response
cfu	Colony forming units
CI	Confidence interval
CFP10	Culture filtrate protein-10
CEF	Cytomegalovirus, Epstein Barr and influenza virus
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulphoxide
DOTS	Directly observed therapy, short-course
ESAT-6	6 kDa early secretory antigenic target
ELISPOT	Enzyme-linked immunosorbent spot
ELISA	Enzyme-linked immunosorbent assay
ELF	Epithelial lining fluid
FCS	Foetal calf serum
FDA	Food and drug administration
FP85A	Fowlpox expressing antigen 85A
HVJ	Haemagglutinating virus of Japan
HPA	Health Protection Agency
HSP	Heat shock protein
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen

Ig	Immunoglobulin
iNOS	Inducible nitric oxide synthase
IFN	Interferon
IL	Interleukin
ICS	Intracellular cytokine staining
LAM	Lipoarabinomannan
LPS	Lipopolysaccharide
LLN	Lung lymph node
MRI	Magnetic resonance imaging
MHC	Major histocompatibility complex
MVA85A	Modified vaccinia virus Ankara expressing antigen 85A
MIF	Monokine induced by gamma interferon
MPL	Mono-phosphoryl lipid A
MDR-TB	Multidrug-resistant tuberculosis
M.	*Mycobacterium*
NK	Natural killer
NRAMP1	Natural resistance-associated macrophage protein 1
NO	Nitric oxide
NTM	Nontuberculous mycobacteria
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PMA	Phorbol 12-myristate 13-acetate
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinin
PVDF	Polyvinylidene difluoride
PPD	Purified protein derivative
RNI	Reactive nitrogen intermediate
ROI	Reactive oxygen intermediate
r	Recombinant
rVV	Recombinant vaccinia virus
RD1	Region of deletion 1
SCID	Severe combined immunodeficiency disease
SIV	Simian immunodeficiency virus
SSI	Statens Serum Institut
TCR	T cell receptor

Th	T helper
TMB	3,3',5,5'-tetramethylbenzidine
TLR	Toll-like receptor
TGF-β1	Transforming growth factor beta 1
TBS	Trizma buffered saline
TST	Tuberculin skin test
TB	Tuberculosis
TNF	Tumour necrosis factor
UK	United Kingdom
VIG	Vaccinia immunoglobulin
WHO	World Health Organisation
XDR-TB	Extensively drug-resistant tuberculosis

Symbols

°C	Degrees Celsius
g	Gravity
h	Hours
<	Less than
µg	Microgram
µl	Microlitre
µm	Micrometre
mg	Milligram
ml	Millilitre
M	Molar
>	More than
ng	Nanogram
nm	Nanometer
%	Per cent
pg	Picogram
P	Probability
n	Sample size
r_s	Spearman's rank correlation coefficient

1 GENERAL INTRODUCTION

1.1 *MYCOBACTERIUM TUBERCULOSIS*

1.1.1 The pathological agent

Tuberculosis (TB) is one of the oldest infectious diseases known to humankind (Salo *et al.*, 1994). It is an infectious disease caused by infection with mycobacteria, mainly *Mycobacterium tuberculosis* (*M. tuberculosis*). It was during a lecture delivered in 1882 that Robert Koch identified the causative agent of TB disease (Koch, 1882). All members of the *Mycobacterium* genus are rod-shaped Gram-positive bacteria that share the property of acid-fastness, due to their mycolic acid-rich cell wall structure (Girard *et al.*, 2005). The cell wall is mainly comprised of a large cell-wall core that contains peptidoglycan, arabinogalactan and mycolic acids. The outer layer, which is generally referred to as the capsule, contains mainly polysaccharides such as glucan and arabinomannan (Abdallah *et al.*, 2007) (Figure 1.1).

Growth of many mycobacteria, in particular the pathogenic species, is slow with a generation time of 12 h for *M. tuberculosis* (Kaufmann & Andersen, 1998). This results in *M. tuberculosis* requiring 3-4 weeks to produce a visible colony (Andersen, 1997).

Since the first encounter between *M. tuberculosis* and man usually happens within the airways, the disease occurs mainly in the lungs, known as pulmonary TB. Successful elimination of the intracellular bacteria depends mainly on the efficient interaction between the infected macropahges and antigen-specific T cells (Stenger & Modlin, 1999).

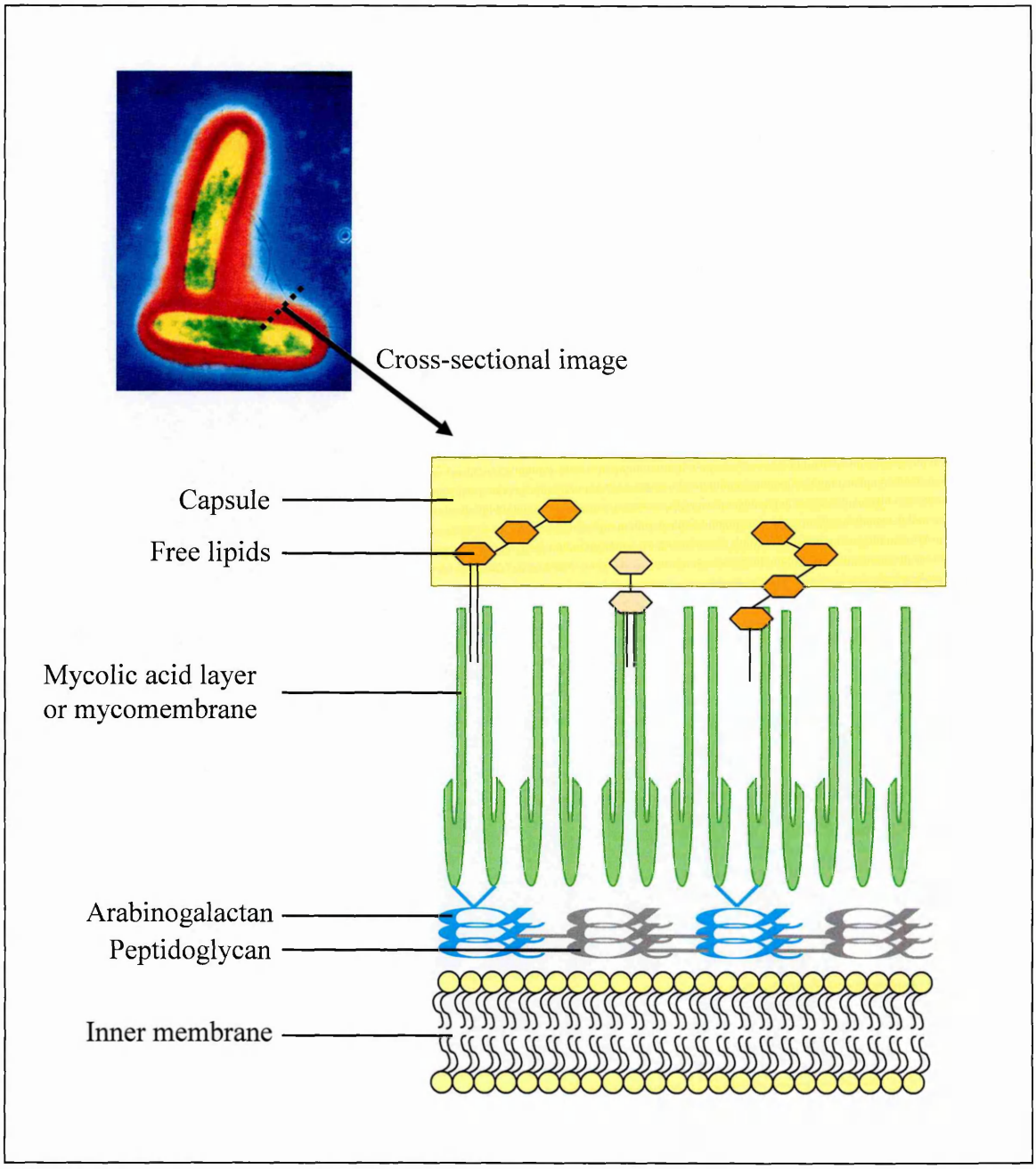


Figure 1.1: Diagram of the mycobacterium cell wall.

(Adapted from Abdallah *et al.*, 2007)

1.1.2 Incidence and prevalence of tuberculosis

During the 10 years preceding 2001, 90 million new TB infections occurred, resulting in approximately 20 million deaths (Collins & Kaufmann, 2001). In 2006, the annual rate in the development of active TB was 8.8 million with 1.6 million deaths resulting from TB disease (WHO, 2006). It has been estimated that one-third to one-half of the world's population is latently infected with *M. tuberculosis* (Kaufmann, 1993). In the vast majority of infected people a long-lasting balance between the host immune response and persistent mycobacteria develops, meaning that the individual remains healthy (Kaufmann, 1993).

Infection with *M. tuberculosis* is generally associated with a 10% life-time risk of clinical disease; however, in human immunodeficiency virus (HIV) co-infected individuals this becomes a 10% annual risk (Young & Stewart, 2002). TB co-infection with HIV has become an issue as both pathogens circulate in similar populations, with disease being chronic and requiring long treatment regimes. Simultaneous treatment of TB and HIV requires 6-10 different drugs (Wells *et al.*, 2007). Both diseases contribute to the pathogenesis of one another; TB accelerates the course of HIV-induced disease by activating viral replication and contributing to the decline in $CD4^+$ T-cell numbers, whereas HIV enhances the risks of reactivating latent TB, increases the progression of a new infection or re-infection to active disease and enables a more rapid spread of strains (Goldfeld & Ellner, 2007).

Treatment against TB is not easy to comply with and can be expensive for countries with limited resources (Sierra, 2006), especially as TB drugs must be taken for a long time. The main antibiotics used in treatment of TB are: isoniazid, rifampicin, pyrazinamide, streptomycin and ethambutol (Sierra, 2006). These are part of the directly observed

therapy, short-course (DOTS) strategy that consists of five elements: political will, case detection, standardised observed therapy, effective drug supply, and monitoring and evaluation (Maartens & Wilkinson, 2007). A combination of at least three antibiotics are required to prevent the emergence of resistance, as a UK national survey in the mid-1950s showed that most drug-resistant strains were only resistant to one antibiotic (Fox *et al.*, 1957). Increasingly though, antibiotic resistant strains are emerging for multiple drugs (Kremer & Besra, 2002), thus compounding the TB peril even further.

Multidrug-resistant TB (MDR-TB) is defined as mycobacteria that are resistant to isoniazid and rifampicin, two of the primary antibiotics used to treat the disease (Matteelli *et al.*, 2007). In 2006 it was estimated that >4% of the global cases of TB were caused by MDR-TB (Zignol *et al.*, 2006). Treatment of MDR-TB is more difficult as the second-line drugs are harder to access and handle, are weaker, have more adverse side effects and are much more expensive, generating treatment costs up to $250,000 (Kaufmann, 2000) compared with approximately $20 per patient for a 6 month standard course of chemotherapy (Matteelli *et al.*, 2007).

More recently, extensively drug-resistant TB (XDR-TB) has been reported where MDR-TB bacilli are also resistant to any fluoroquinolone and at least one of three injectable second-line drugs (Jain & Mondal, 2008). The worldwide prevalence of XDR-TB is reported to be 6.6% among all MDR-TB isolates (Jain & Mondal, 2008).

In order to control TB it is apparent that further control measures are needed. New antibiotics against MDR-TB are being developed and tested (reviewed in (Dover *et al.*, 2008), but there have been few new anti-tubercular drugs introduced into widespread use since rifampicin in the 1960s (Kremer & Besra, 2002). An example of a new anti-TB drug which has demonstrated strong potential is a diarylquinoline, named R207910, that

blocks the function of an essential membrane-bound enzyme that makes adenosine triphosphate (Sharma & Mohan, 2006). This compound has been shown to be bactericidal with specific activity against mycobacteria, including MDR-TB strains (Andries *et al.*, 2005), and has entered clinical trials (Matteelli *et al.*, 2007). However, it can be hypothesized that mycobacteria will develop resistance against any new drugs introduced, in much the same way as resistance has developed against the other antimycobacterial compounds. For example, in Italy two patients were diagnosed with TB that was resistant to all known anti-TB drugs (Migliori *et al.*, 2007). Therefore, unless treatment against TB is improved and control measures successfully implemented, then the development of drug resistance is expected to continue (Fauci, 2008) for current agents, and perhaps future ones too.

Generating immunity against *M. tuberculosis* may prove to be a more long-term and successful approach that circumvents the issues of antibiotic resistance. Additionally, vaccines that confer protection against TB disease will eliminate the requirement for extensive chemotherapeutic treatment. Another advantage with vaccines is that they are administered at only a few timepoints, whereas treatment against TB disease requires continual administration of anti-TB drugs and monitoring lasting several months, so vaccination is far more convenient and economical. The only currently available licensed vaccine against TB is the bacille Calmette Guérin (BCG) vaccine.

1.2 VACCINATION AGAINST TUBERCULOSIS

1.2.1 BCG vaccine: history and effectiveness

In the 1920s, two students of Louis Pasteur, Calmette and Guérin, succeeded in attenuating *M. bovis* by sequential culture on potato-bile broth (Roche *et al.*, 1995). This process repeated 230 times over 13 years yielded a strain of bacillus, *M. bovis* bacille Calmette Guérin, which had no pathogenicity in guinea-pigs or cattle (Roche *et al.*, 1995). This attenuated strain was subsequently used as a vaccine against TB, and still remains the only licensed vaccine against this disease.

It is estimated that 76% of children worldwide are vaccinated with BCG, with meta-analysis of 18 case-control studies revealing efficacies of 73% and 77% against childhood TB meningitis and miliary TB, respectively, in the first 5 years of life (Trunz *et al.*, 2006). However, while BCG is very effective against childhood and disseminated TB, it is less effective against adult pulmonary TB (Roche *et al.*, 1995). In the general population an average efficacy of 50% (0-80%) was reported in a meta-analysis of the literature covering 1264 articles from which 70 clinical trials were selected spanning a 46 year period (Colditz *et al.*, 1994). These extremes in variation of protection against pulmonary TB are highlighted by experiments reported in the 1970's where extensive trials in India showed a protective efficacy of 0% (Tuberculosis Prevention Trial, 1979; Tuberculosis Research Centre, 1999), whereas in the UK trial efficacies of 78% were observed (MRC, 1972). The causes of these variations may include inherent deficiencies of the BCG vaccine, or by differences in the study populations, particularly in regard to pre-existing immune responses in those individuals where BCG failed to protect against TB.

1.2.2 Limitations of the BCG vaccine

There is evidence that the immunity induced by BCG is not life-long. From a meta-analysis of trials, there is no strong evidence that BCG provides protection for more than 10 years after vaccination (Sterne *et al.*, 1998). Contrary to these results, studies in North American Indians and Alaskan Natives have demonstrated that there was only a slight decrease in the protective efficacy of BCG vaccination after 50 years of observations (Aronson *et al.*, 2004).

By the 1980s, it was apparent that BCG from different localities had evolved into strains with some distinct antigenic differences (Roche *et al.*, 1995), with over 16 separate strains of BCG vaccine available worldwide (Behr *et al.*, 1999). Major BCG vaccine strains differ from the original BCG strain and from each other, with "stronger" strains (Pasteur 1173, Danish 1331) being more reactogenic and, presumably, more immunogenic, than "weaker" strains (Glaxo 1077, Tokyo) (Girard *et al.*, 2005). However, even when the same strain (Danish) was used, there was good protection in the UK but poor protection in India (Roche *et al.*, 1995). A meta-analysis of human BCG vaccination with different strains also found that the strain of BCG was not a significant determinant of the overall efficacy in the prevention of TB (Brewer & Colditz, 1995).

Over time, BCG vaccines may have lost a number of genes with potential relevance for protective immunity or, in other words, have been "gradually attenuated to impotence" as seen by reduced efficacies with increasing passage number (Behr & Small, 1997). With over 100 genes deleted in BCG compared to *M. tuberculosis* (Behr *et al.*, 1999) the vaccine lacks important immunodominant antigens expressed in virulent mycobacteria, such as 6kDa early secreted antigenic target (ESAT-6) (Harboe *et al.*, 1996). It could be that some of these antigens will be needed to mount an effective immune response

against *M. tuberculosis.* However, losing some of the virulence genes also benefits the use of BCG as it contributes to the bacilli being made into a safe vaccine.

1.2.3 Pre-existing immunity and the effect on BCG vaccination

Protective immunity from BCG requires bacterial replication in the host leading to the generation of mycobacterial immunity. This protective immune response may be blocked by pre-existing immunity cross-reacting to BCG (Dietrich *et al.*, 2006). Environmental mycobacteria are ever-present in nature, and contact between them and humans and animals is therefore a frequent and regular occurrence (Zumla & Grange, 2002). One of the areas where BCG is ineffective as a vaccine is in Malawi, South-Eastern Africa. Using six atypical mycobacterial strains isolated from soil and sputum samples in Malawi, two strains of *M. avium* were found to block BCG protective efficacy completely in mice (Brandt *et al.*, 2002). A large study undertaken in Malawi also demonstrated patterns of immune response against *M. tuberculosis* purified protein derivative (PPD) in healthy, HIV-seronegative, Malawian young adults with no history or scar evidence of prior BCG vaccination (Black *et al.*, 2001). These results suggest that an appreciable proportion of the individuals living in this rural area of northern Malawi had been exposed to mycobacterial antigens by the age of 12, and that such responses increase with age (Black *et al.*, 2001). Exposure to *M. avium* in the Malawian population was much more frequent compared to *M. tuberculosis* and *M. bovis* as assessed by using PPD preparations from the different mycobacterial species (Black *et al.*, 2003). These data suggest that individuals living in Malawi may be less able to generate protective immune responses to BCG than their UK counterparts (Bennett *et al.*, 2006) due to prior sensitisation with environmental mycobacteria blocking subsequent vaccine responses. However, in UK school children who had not been BCG-vaccinated, IFN-γ responses were detected to PPD derived from *M. avium* (60% responding) and *M. tuberculosis*

(27% responding), indicating that this population also had background responses from pathogenic and environmental mycobacteria (Weir *et al.*, 2003).

Based on animal studies, it has been suggested that infection with environmental mycobacteria changed the immune reaction towards a detrimental humoral response that could not be overridden by a subsequent BCG vaccination (Hernandez-Pando *et al.*, 1997). Another argument is that in areas where BCG fails to work, people have concurrent infections with helminths. Individuals with helminth infections are chronically immune-activated and also have pronounced humoral immune profiles (Borkow & Bentwich, 2000), thus likely effecting BCG vaccination responses which require T cell induced immunity.

With the evidence that BCG protects against disseminated disease in childhood and confers reliable protection against leprosy (Ponnighaus *et al.*, 1992), until it is clearly demonstrated that a replacement vaccine can confer an equal level of protection to BCG it would be unethical to withdraw BCG vaccination (Fletcher & McShane, 2006). With roughly one case of severe childhood tuberculosis being prevented for every 2500 BCG inoculations (Novelli, 2006) there is still extreme value in immunising children against TB disease in areas with high endemnicity.

As BCG vaccination was thought to be a success in the eradication of TB, it has been said that, "Research on tuberculosis and BCG seems to have suffered from its own early success; that is, it seems as though the global BCG campaigns were more successful in eradicating tuberculosis research than in eradicating tuberculosis" (Fine, 1989). However, historical evidence has demonstrated that improvements on socio-economic conditions have contributed more to the decline in TB in the last century than have medical interventions (Grange, 1999). This is also reflected by the fact that developing

countries attribute to 97% of TB cases and 98% of TB deaths in the world (Benatar, 2003).

With the realisation that the current BCG vaccine will not eradicate TB, there has been considerable interest in developing new vaccines to replace, or enhance, the BCG vaccine.

1.2.4 New TB vaccine candidates

A large number of potential candidates are being evaluated in the search for a new vaccine against TB. These vaccines are based on different approaches: (i) subunit vaccines; (ii) live attenuated vaccines; and (iii) combination vaccines. These are discussed below, with examples given of the main candidates currently at the furthest stage of development.

1.2.4.1 Subunit vaccines

Subunit vaccination assumes that one or only a few antigens are required to generate protective immunity (Kaufmann, 2000). However, the immune response may be limited in the required intensity and duration, so subunit vaccines may require the addition of an adjuvant formulation (Sierra, 2006) or prior vaccination with BCG.

1.2.4.1.1 Protein subunit vaccines

A fusion protein of Ag85B-ESAT-6 has shown protection in the guinea-pig model (Olsen *et al.*, 2004). This vaccine has been shown to confer protection in cynomolgus

macaques, as assessed by reduced bacterial load and lung pathology (Langermans *et al.*, 2005). Also in cynomolgus macaques, the protein was coupled with a phosphoantigen and after vaccination two waves of immune response were observed successively by γδ and αβ T lymphocytes (Cendron *et al.*, 2007). However, this vaccine may suffer because of the inclusion of ESAT-6 which may compromise the specificity of new immunodiagnostic tests (Sander & McShane, 2007) which use ESAT-6-specific responses to diagnose TB infection.

Ag85 complex proteins, when formulated with adjuvant and delivered intranasally, have been shown to impart protection comparable to that of BCG when delivered in mice (Giri *et al.*, 2006). Also, Ag85A protein has been administered subcutaneously to boost BCG immune responses in mice, thus extending the protection against aerosol *M. tuberculosis* infection into later age (Brooks *et al.*, 2001). Priming mice with Ag85A DNA before vaccinating with BCG increased significantly the protective efficacy of the BCG vaccine (Romano *et al.*, 2006).

1.2.4.1.2 *Vector-based subunit vaccines*

Mycobacterial antigens have been engineered into non-mycobacterial bacteria such as *Salmonella* (Hess & Kaufmann, 2001), which can induce strong mucosal responses (Sierra, 2006). Viral vectors have also been used (Goonetilleke *et al.*, 2003), which have the potential to induce cytotoxic T-lymphocyte (CTL) responses (Sierra, 2006). However, vaccines that have been attenuated but are still live will require critical safety evaluation, especially for use in immunodeficient populations (Sierra, 2006). Vaccines that use replication incompetent organisms to deliver antigens would offer an easier route through the regulatory process.

Recombinant pox-viruses and recombinant adenoviruses are efficient at boosting previously primed T cell responses as these vectors induce non-specific co-stimulated responses that facilitate the amplification of pre-existing memory immune responses (McShane & Hill, 2005). Ag85A has been expressed in numerous viral vectors, including Modified Vaccinia Ankara (MVA) (Goonetilleke *et al.*, 2003), fowlpox (Williams *et al.*, 2005a), vesicular-stomatitis virus (Xing & Lichty, 2006) and adenovirus (Xing & Lichty, 2006). In mice, MVA85A was shown to boost BCG-induced Ag85A specific $CD4^+$ and $CD8^+$ T cell responses (Goonetilleke *et al.*, 2003). When MVA85A and fowlpox expressing Ag85A (FP85A) were used in the guinea pig model to boost BCG vaccination responses, the poxvirus-boosted animals gave 100% protection up to 26 weeks post-*M. tuberculosis* high-dose challenge (Williams *et al.*, 2005a). In phase I human clinical trials, MVA85A boosting of BCG vaccination responses was found to induce high levels of antigen-specific IFN-γ-secreting T cells (McShane *et al.*, 2004). The BCG-MVA85A prime-boost regime is currently being tested in phase II clinical trials in South Africa (Pathan *et al.*, 2007), where is has been shown to be safe and immunogenic (Hawkridge *et al.*, 2008).

Ag85A inserted into an adenovirus vector (AdAg85A) has been used as a boost vaccine after BCG vaccination in mice (Santosuosso *et al.*, 2006). However, only when AdAg85A was delivered intranasally was protection observed (Santosuosso *et al.*, 2006). Other investigators have inserted genes encoding *M. tuberculosis* antigens Ag85A, Ag85B, and TB10.4 into an adenovirus vector (Ad35-TBS). When this vaccine was used without prior BCG vaccination, mice were protected against an *M. tuberculosis* challenge (Radosevic *et al.*, 2007).

1.2.4.2 Live attenuated vaccines

Whole bacterial vaccines contain many antigens to enhance protection and benefit from having adjuvanticity properties (Kaufmann, 2000). These vaccines may induce long-lasting immune responses, but may be disadvantaged in having the potential to cause immunopathology (Sierra, 2006). Multiple mutations are essential to ensure that there is no potential for the vaccine organism to revert to virulence (Young & Stewart, 2002). The two main approaches used for live attenuated vaccines against TB include using BCG and *M. tuberculosis* bacilli.

1.2.4.2.1 Attenuated BCG vaccines

A BCG strain with the urease gene deleted and a listeriolysin gene from *Listeria monocytogenes* inserted has shown greater potential than the parental BCG strain in a mouse model (Grode *et al.*, 2005). The lack of urease allows the phagosome to acidify, and the listeriolysin enables the phagosome to be punctured; allowing passage of the organism into the cytoplasm and access to MHC class I molecules. Another live attenuated BCG vaccine uses BCG overexpressing antigen 85B, a mycolyl transferase involved in mycobacterial cell wall synthesis, which has shown protection in guinea pigs (Horwitz & Harth, 2003) and has completed a phase I clinical trial (Skeiky & Sadoff, 2006).

Ag85A has also been introduced into a recombinant form of BCG which, when boosted with Ag85A peptide, improved lung pathology and reduced pulmonary bacterial load significantly in guinea pigs after infection (Sugawara *et al.*, 2007b). This vaccine

approach has also been assessed in non-human primate models (Sugawara *et al.*, 2007a; Sugawara *et al.*, 2009).

1.2.4.2.2 Attenuated M. tuberculosis vaccines

An advanced live attenuated vaccine that uses *M. tuberculosis* is a strain with a disruption in the *phoP* gene, which regulates mycobacteria lipids implicated in the virulence of *M. tuberculosis* (Ludwiczak *et al.*, 2002; Walters *et al.*, 2006) called SO2. Recently, it has been shown that the *phoP* gene controls secretion of the major antigens ESAT-6 and CFP10 (Frigui *et al.*, 2008). This SO2 strain has been used as a vaccine in mice, where protection against challenge with *M. tuberculosis* was similar to BCG vaccinated animals (Aguilar *et al.*, 2007). Furthermore, *M. tuberculosis* SO2 has been tested in guinea pigs and severe combined immunodeficiency disease (SCID) mice, demonstrating better protection against a high-dose challenge in guinea pigs compared to BCG vaccination and safety in immunocompromised subjects (Martin *et al.*, 2006).

1.2.4.3 Combination vaccines

Instead of using a single vaccine to control *M. tuberculosis*, a combination of vaccinations may prove essential in order to allow sufficient immunity to be generated. One such approach uses two different vaccines - each containing the same antigen – to allow a directed response against this antigen to be developed; so-called 'heterologous prime-boost' (McShane & Hill, 2005). Instead of ignoring previous BCG inoculation, as BCG vaccination is routinely administered to the young in countries where tuberculosis is endemic, targeting an antigen present in BCG that has some memory T cell activity has the potential to induce a fresh immune response (Brooks *et al.*, 2001). As Ag85A is

conserved amongst all mycobacterial species and is present in all strains of BCG (D'Souza *et al.*, 2003) this antigen has been identified as an ideal candidate for boosting BCG vaccination responses. As mentioned in section 1.2.4.1.2, the advanced MVA85A vector vaccine delivered after BCG vaccination is currently in human clinical trials.

Boosting BCG responses with proteins has also successfully been tested. Mtb72F, a fusion protein of Mtb39 (PPE protein) and Mtb32 (serine protease) administered with a CD4 T-cell adjuvant (ASO2A), has shown protective efficacies greater than using BCG alone in guinea pigs (Brandt *et al.*, 2004). This vaccine has also completed clinical trials in the United States and Europe (Sander & McShane, 2007).

1.3 ANIMAL MODELS OF TUBERCULOSIS

As immunological correlates of protection have yet to be identified in any animal models of TB, the only way to assess the protective efficacy of new vaccines is to conduct challenge studies (McMurray, 2000b; 2001b; McMurray *et al.*, 1999). The use of relevant animal models in TB research is therefore critical to allow the onward development and testing of new vaccines and treatments. These animal models should ideally produce levels of infection and disease at high prevalence, yet the pathogenesis of disease that occurs after artificial infection must mimic disease patterns that occur naturally (Griffin, 2000). Animal species that contract TB following natural exposure to *M. tuberculosis* provide representative experimental model systems which are most relevant for the study of human disease (Griffin *et al.*, 2001). Animal models for tuberculosis have been used since 1882, when Robert Koch first described the causative agent backed up by experimental infection in animals including mice, rats, guinea pigs, hamsters, cats and dogs (Koch, 1882). Since then, in excess of 100 tuberculosis vaccines have been tested in animal models (Girard *et al.*, 2005).

Although it is acknowledged that animal models of *M. bovis* infection closely resemble events with *M. tuberculosis* infection, due to the latter organism being used for this project only this is introduced in this section.

1.3.1 Murine models

The mouse provides a sophisticated and cost-efficient model for TB disease. The inbred mouse is the most popular model in TB research, and has provided a large amount of information in terms of understanding mechanisms of immunity that are relevant to humans (Orme, 2003).

Mice are more resistant than humans to infection with *M. tuberculosis*, and the disease process differs significantly from human TB. The granulomas of mice rarely progress to necrosis, caseation and liquefaction as they often do in humans and they are able to sustain very high levels of mycobacteria in their lungs for months without apparent disease (Kremer & Besra, 2002). Using the low-dose aerosol model of infection it has become clear that certain inbred mouse strains differ in their susceptibility to *M. tuberculosis* infection. Certain strains (e.g. DBA/2, A/J, CBA/J) are unable to control infection in the lungs and subsequently show evidence of a form of reactivation or regrowth that is fatal to the animal (Orme, 2003). In contrast, mice on the C57 genetic background maintain a chronic bacterial load (Orme, 2003).

The resistant nature of the mouse model makes it a suitable animal in which to study latency. The Cornell model can also be used to mimic latency and involves treating *M. tuberculosis* infected mice with antimycobacterial drugs to reduce the bacterial burden to undetectable levels. Reactivation of disease can then occur spontaneously or in response

to immunosuppressive agents (Flynn & Chan, 2001b). The advantage of this latency model is the low bacterial burden in mice; however, the introduction of antibiotics does not replicate the situation in natural human latent tuberculosis and may influence the development of a protective immune response.

To try to recreate the slow progression from *M. tuberculosis* infection to disease, as usually found in humans, the intraperitoneal route of infection has shown some signs of success (Mustafa *et al.*, 1999). Additionally, to try and recreate the intragranulomatous lung necrosis that the mouse model usually lacks, but in human disease commonly develops, lipopolysaccharide (LPS) has been inoculated via the intranasal route in *M. tuberculosis* infected mice (Cardona *et al.*, 2001). This provided a feasible and reproducible tool to recreate the histopathology of human TB in the murine model.

As studies involving animal models of TB need to be carried out in Containment Level 3 facilities, the mouse has a clear advantage in this regard as many more mice than guinea pigs or rabbits can be housed in the same space, and the cost of maintaining mice is much less (McMurray, 2000b). However, the small size also means that there are differences in the mouse model compared to humans. The bronchial tree is far less complex in mice, and there are differences in the structure, such as no second circulatory supply to bronchial associated lymphoid tissue. As the lymphatics are less complex in mice, with limited drainage into the alveolar regions (Orme, 2003), the development of immunity and presentation of antigens will be different to those occurring during human infection.

Preliminary screening of TB vaccines in mice is a cost-effective strategy, and challenging small animals with aerosolised organisms has been accepted to be one of the first stages in evaluating potential vaccines against mycobacteria (Skinner *et al.*, 2001).

Large numbers of candidates can be narrowed down to the most promising few, that then can undergo secondary screening in larger animal models (Orme, 2003). One such example is the fusion protein 72f, that has been found to have protective effects in the guinea pig and primate models after its activity was initially observed in the mouse (Orme, 2003). However, mice are not infallible in predicting efficacy in larger animal species. While DNA vaccination has proven to be an efficient immunisation strategy in mice, it has been shown to be less effective in larger animals so further improvements will need to be made before DNA can be used in human vaccination (Reed *et al.*, 2003).

Effects of prior exposure to mycobacteria studied in the mouse model have demonstrated that sensitisation with environmental mycobacteria inhibited BCG multiplication and thereby prevented the induction of an efficient BCG-mediated response and subsequent protection against *M. tuberculosis* challenge (Brandt *et al.*, 2002). This experimental model is therefore relevant to the many tropical regions where BCG is not protective against pulmonary TB.

A lot of our understanding of the T cells involved in protection against *M. tuberculosis* (covered in section 1.4) is based on evidence from studies of TB in mice (Mogues *et al.*, 2001). Work on murine immunology is greatly assisted by the vast number of reagents that are available for this model, including monoclonal antibodies to T cell surface markers, primers and probes for the growing array of cytokines and chemokines involved, and the availability of inbred mouse strains (Orme, 2003). Mice generate a strong cellular response, which controls bacterial growth and limits damage to the lungs (Baldwin *et al.*, 1998) and extensive studies carried out using TB vaccines in inbred mice have identified a variety of responses that may be involved in protective immunity following vaccination.

Although mice constitute an excellent model to monitor fundamental parameters of immunity, they lack some of the components that are present in humans, e.g. group I CD1 molecules (Kaufmann, 2001) and granulysin (Lazarevic & Flynn, 2002). Therefore, when the contribution of these components need to be studied, mice are not the most appropriate models to use.

1.3.2 Guinea pig models

The guinea pig is an invaluable animal model of human TB, the central reason being the fact that guinea pigs develop 'classical' granulomas similar to those seen in humans with active disease (Reed *et al.*, 2003). This provides a distinct advantage over the mouse model, where this phenomenon is absent, whilst maintaining the benefit of being a small animal.

After infection with low doses of virulent *M. bovis* or *M. tuberculosis* guinea pigs are particularly susceptible to progressively fatal disease (Griffin, 2000); infection in guinea pigs can result from just a single virulent organism (McMurray, 2001c). This may be a result of the animal initially developing a strong immune response, which eventually leads to considerable tissue damage resulting in extensive caseation and tissue necrosis that leads to death (Baldwin *et al.*, 1998). These processes mimic responses in infected humans, and so the guinea pig provides a useful model to study these events.

The series of events following *M. tuberculosis* aerosol challenge in guinea pigs has been well documented (Balasubramanian *et al.*, 1994a). The first event after delivery of a low aerosol challenge dose is the development of one or more primary lesions in the pleural surface of the lungs. Following this, the virulent bacilli multiply at the site of implantation in the lung, and some bacilli get transported to the lymph nodes draining

that specific region of the lungs. The bacilli then multiply in the lymph node which enlarges, creating a situation in which some bacilli escape and enter the bloodstream, to be observed later in the spleen and in other lung lobes (Balasubramanian *et al.*, 1994a).

The aerosol challenge of guinea pigs has been used extensively to evaluate novel TB vaccines (Vipond *et al.*, 2006a; Vipond *et al.*, 2006b; Williams *et al.*, 2005b). As BCG vaccination protects well in the guinea pig model (Williams *et al.*, 2000), it may prove difficult for a candidate vaccine to improve upon this performance. Successful immunisation of guinea pigs leads to reduced necrosis, with small lesions characterised by infiltrating lymphocytes, decreased weight loss and prolonged survival. Recently, more rigorous vaccine evaluations in guinea pigs have tended towards using disease and survival as endpoints instead of only short-term bacterial counts, which are not necessarily predictive of long-term disease outcome (Baldwin *et al.*, 1998; Reed *et al.*, 2003).

Immunological studies on guinea pigs is restricted by the lack of available reagents and due to their small size, sequential sampling of blood is difficult in this model. However, several guinea pig cytokine and chemokine genes have now been cloned, so reagents are being developed to address this requirement and allow the study of immunological determinants of resistance to TB in this model (McMurray, 2001c), including guinea pig micro-arrays (Tree *et al.*, 2006) and antibodies to IFN-γ (Schafer *et al.*, 2007). This will greatly enhance the use of the guinea pig model and allow the generation of important immunological data.

1.3.3 Rabbit model

Although the rabbit is more costly compared to the mouse and guinea pigs models in terms of purchase, maintenance, and the amount of high containment space required, it does reproduce the range of pathological consequences seen in human TB (McMurray, 2001c). Although rabbits are highly susceptible to infection with virulent *M. bovis*, they are much more resistant to infection with *M. tuberculosis* (McMurray, 2001c).

Pulmonary granulomas in rabbits often undergo liquefaction, so this model allows the process of lung cavitation to be studied, which is important as cavitation is also observed in human TB disease. Tubercle bacilli may grow extracellularly in these cavities, from where bacilli can enter the bronchial tree and spread to other parts of the lung and also to other people (Converse *et al.*, 1996). The spread to other people is usually through the coughing mechanism which distributes the bacilli around the patient's environment. Therefore, liquefaction and cavitation are important implications that enable the spread of TB (Converse *et al.*, 1996).

Pulmonary TB in rabbits, therefore, represents an excellent model for pulmonary TB in humans. Neither species is inbred, so considerable variation in the amount and extent of disease occurs. These factors make studies of both rabbits and humans more difficult and more expensive, but cavitary TB cannot be produced in mice and only rarely in guinea pigs (Converse *et al.*, 1998).

To further analyse information from this model, cytokine reagents are being developed for the rabbit (McMurray, 2001c) to allow more immunological analyses to be carried out. However, mainly due to its size, the model is limited in its use as a widely available vaccine testing model, and is instead used more prominently as a model for the study of cavitary disease formation.

1.3.4 Non-human primate models

1.3.4.1 Introduction

Non-human primates are susceptible to tuberculosis infection by the aerosol route, develop a human-like disease, exhibit antigen-induced T lymphocyte reactivity both *in vitro* and *in vivo*, and can be protected effectively by BCG vaccination (McMurray, 2000a). The similarity in responses may be because host molecules are present in both man and non-human primates, but differ in mice and guinea pigs (Langermans *et al.*, 2001). In their natural habitats, non-human primates are essentially free from tuberculosis (Mulder, 1976) so the infection is primarily induced through human intervention.

The course of *M. tuberculosis* infection in macaques can be followed by chest X-ray and weight loss, as well as by a variety of haematological tests, including erythrocyte sedimentation rate, lymphocyte proliferation, cytotoxic T-lymphocyte activity and cytokine production (Reed *et al.*, 2003). The ability to perform detailed analyses of both pathology and immune responses demonstrates the potential of non-human primates in TB vaccine development.

Due to the large size of the non-human primates, sequential blood sampling can be undertaken to assess longitudinal responses in the same animal. Macaque blood has been used successfully in modern immunological assays, such as the whole blood cytokine flow cytometry methodology (Keeney *et al.*, 2003) and enzyme-linked immunosorbent spot (ELISPOT) assays (Makitalo *et al.*, 2002). Results from these assays allow comparison with data obtained from human studies. Additionally, the role of $CD4^+$, $CD8^+$ and γδ cell populations can be studied in this model (Lai *et al.*, 2003; Shen *et al.*, 2002).

There are some difficulties in using non-human primates for research; the major factors being containment of biohazards and cost. Experimentation with airborne infections in primates imposes two major requirements: animals must receive known dosages of the infecting agent and secondly, the exposure and subsequent handling of the animals should present a minimal hazard to those carrying out the work (Wolochow *et al.*, 1957). Additionally, although the outbred nature of macaques can be viewed as a limitation on performing some studies, this also likely contributes to the wide range of clinical manifestations seen in this model (Capuano *et al.*, 2003), so this resemblance to humans may in fact prove to be an advantage.

Because of their similarities to humans, non-human primates represent the best animal models for assessing the relative safety and immunogenicity of vaccines (Pehler *et al.*, 2000). Non-human primates also demonstrate many comparable gross and microscopic pathological changes consistent with the different disease stages in people (Capuano *et al.*, 2003). The macaque model can simulate various important aspects of human TB, including primary disease as well as latent infection (Flynn *et al.*, 2003; Gormus *et al.*, 2004). However, primate testing should be reserved for the final stages of evaluation of vaccine candidates that have already shown significant protective activity in smaller

animal models (McMurray, 2000a), and should be used to supplement data from these animals, not to replace them (Schmidt, 1972).

It is also possible to co-infect macaques with other pathogens in order to produce models of infection that are similar to situations found in humans, for example, using simian immunodeficiency virus (SIV) as a model for human immunodeficiency virus (HIV) infection. Results from SIV/BCG co-infected monkeys have confirmed the hypothesis that mycobacterial coinfection can enhance viral pathogenicity as well as accelerate the progression to clinical AIDS in HIV-infected people (Zhou *et al.*, 1999).

There have been some studies reported on the use of baboons (*Papio cynocephalus anubis*) in the areas of immunogenicity of *M. tuberculosis*-secreted protein antigens (Pehler *et al.*, 2000) and during an outbreak of disease in a captive colony (Tribe & Welburn, 1976). However, most non-human primate tuberculosis work has been carried out in the rhesus and cynomolgus macaque models (tables 1.1 and 1.2, respectively). Rhesus macaques are generally heavier (average adult size 9509g) compared to cynomolgus macaque (5454g) (Andrade *et al.*, 2004). Both animals have similar crown-to-rump lengths (rhesus macaques, 46.53cm; cynomolgus macaques, 41.25cm), but tails are far larger in adult cynomolgus macaques (54.79cm) than in the rhesus macaques (24.20cm) (Andrade *et al.*, 2004). These figures are from 175 rhesus macaques and 36 cynomolgus macaques housed for biomedical research, and are from adult macaques defined as being aged over 45 months.

Year	Macaque species (number)	Vaccination/Purpose of study	Challenge	Reference
1956	Rhesus (221)	Assess treatment with isoniazid	Naturally infected beforehand	(Schmidt, 1956)
1968	Rhesus (>1300)	BCG and mycobacterial cell walls (intravenously)	*Mtb* 5159 strain via bronchus instillation (145-500 viable units)	(Good, 1968)
1970	Rhesus (x26)	BCG (intravenously)	*Mtb* H37Rv via aerosol (12-49 infectious units)	(Barclay *et al.*, 1970)
1971	Rhesus (x40)	Mycobacterial cell walls and BCG (intravenously)	*Mtb* H37Rv via aerosol (21 infectious units)	(Ribi *et al.*, 1971)
1972	Rhesus (59)	BCG and BCG cells walls (intramuscularly and intravenously)	*Mtb* H37Rv via aerosol (62 units)	(Anacker *et al.*, 1972)
	Rhesus (~417)	BCG (intracutaneously, intravenously and intratracheal)	*Mtb* H37Rv and strain 5159 & 4941 via bronchial instillation (50-5000 viable units)	(Schmidt, 1972)
1973	Rhesus (x55)	BCG (aerosol and intracutaneously)	*Mtb* H37Rv via aersol (11.6-16.5 infectious units)	(Barclay *et al.*, 1973)
	Rhesus (x50)	BCG (intravenously and intracutaneously)	*Mtb* H37Rv via aerosol (14 viable units)	(Janicki *et al.*, 1973)
2000	Rhesus (x4)	Culture filtrate proteins (intramuscular)	ND	(Attanasio *et al.*, 2000)
2001	Rhesus (x6)	BCG (intradermally)	*Mtb* Erdman via intratracheal instillation (3000 cfu)	(Langermans *et al.*, 2001)
2002	Rhesus (x8)	BCG (intravenously)	*Mtb* H37Rv via aerosol (400-500 cfu)	(Shen *et al.*, 2002)
2003	Rhesus (x20)	BCG (intravenously and bronchial inoculation)	ND	(Lai *et al.*, 2003)
2004	Rhesus (x20)	ND (pathogenesis study)	*Mtb* Erdman and H37Rv via intrabronchial inoculation (10-2x10^6 cfu)	(Gormus *et al.*, 2004)
2008	Rhesus (x15)	rBCG expressing perfringolysin and rAd expressing Ag85A, Ag85B and TB10.4.	ND	(Magalhaes *et al.*, 2008)
	Rhesus (x4)	ND (pathogenesis study)	*Mtb* H37Rv via aerosol (400-500 cfu)	(Huang *et al.*, 2008)
2009	Rhesus (x15)	rBCG-Ag85A	*Mtb* H37Rv via intratracheal instillation (3000 cfu)	(Sugawara *et al.*, 2009)

Table 1.1: Summary of tuberculosis vaccine and pathogenesis studies carried out in rhesus macaques

(ND = Not done, *Mtb* = *M. tuberculosis*, cfu = colony forming units).

Year	Macaque species (number)	Vaccination/Purpose of study	Challenge	Reference
1996	Cynomolgus (x24)	ND (pathogenesis study)	*Mtb* Erdman via intratracheal instillation (10-10^5 cfu)	(Walsh *et al.*, 1996)
2001	Cynomolgus (x6)	BCG (intradermally)	*Mtb* Erdman via intratracheal instillation (3000 cfu)	(Langermans *et al.*, 2001)
2003	Cynomolgus (x17)	ND (pathogenesis study)	*Mtb* Erdman via bronchoscope instillation (~25 cfu)	(Capuano *et al.*, 2003; Flynn *et al.*, 2003)
	Cynomolgus (x9)			(Fuller *et al.*, 2003)
2005	Cynomolgus (x12)	Ag85B-ESAT-6 fusion protein	*Mtb* Erdman via intratracheal instillation (1000-3000 cfu)	(Langermans *et al.*, 2005)
2006	Cynomolgus (x8)	ND (pathogenesis study)	*Mtb* Erdman by bronchoscopic instillation (25 cfu)	(Lin *et al.*, 2006)
2007	Cynomolgus (x16)	ESAT-6-Ag85B fusion protein +/- phosphoantigen	ND	(Cendron *et al.*, 2007)
	Cynomolgus (x18)	rBCG-Ag85A	*Mtb* H37Rv by intratracheal instillation (3000 cfu)	(Sugawara *et al.*, 2007a)
2008	Cynomolgus (x3)	ND (pathogenesis study)	*Mtb* Erdman via bronchoscope instillation (1000 cfu)	(Huang *et al.*, 2008)

Table 1.2: Summary of tuberculosis vaccine and pathogenesis studies carried out in cynologus macaques

(ND = Not done, *Mtb* = *M. tuberculosis*, cfu = colony forming units).

1.3.4.2 Rhesus macaques

Since 1956, rhesus macaques have been regarded as suitable models for carrying out controlled studies on new agents for the treatment of human TB (Schmidt, 1956). Early vaccine experiments on non-human primate TB research from the 1970s utilised only the rhesus model. Most of the experiments focused on the effects of vaccination with BCG including using different routes of immunisation (Barclay *et al.*, 1970; Barclay *et al.*, 1973; Janicki *et al.*, 1973) and the use of mycobacterium cell walls as a vaccine (Anacker *et al.*, 1972; Ribi *et al.*, 1971; Schmidt, 1972). These experiments showed that BCG provided protection against *M. tuberculosis* challenge. After 1973, there were no published experiments using rhesus macaques for over 25 years.

Recent experiments have included studies on the immunogenicity and safety of *M. tuberculosis* culture filtrate proteins (Attanasio *et al.*, 2000) and models of asymptomatic TB (Gormus *et al.*, 2004). The latter study showed that asymptomatic TB has similarities to latent tuberculosis and is of particular significance as rhesus macaques are also the best characterised models for human AIDS research. Therefore, the rhesus macaque provides the best model for the studies of interactions between TB and SIV disease. With one-third of the world's population being infected with *M. tuberculosis* (Kaufmann, 1993), this organism represents the leading cause of death in HIV-infected persons (Medaglini & Hoeveler, 2003) so these co-infection studies are of utmost importance.

Although the rhesus macaque model does not always predict accurately the actions and toxicities of drugs and biological agents for man, in the 1970s it was reported to have an "enviable record for predictability better than that of any other laboratory animal" (Schmidt, 1972). It has also been suggested the rhesus macaque may be best used for

efficacy studies on improved live attenuated vaccines that may show a clear improvement over BCG vaccination (Langermans *et al.*, 2001). However, this was suggested from a study where BCG vaccination in rhesus macaques was unsuccessful, which is in contrast to other reports where BCG conferred protection against *M. tuberculosis* infection (Anacker *et al.*, 1972; Barclay *et al.*, 1970; Barclay *et al.*, 1973; Janicki *et al.*, 1973; Ribi *et al.*, 1971; Schmidt, 1972).

1.3.4.3 Cynomolgus macaques

Low dose infection of cynomolgus macaques can provide a relevant model for the study of pathogenesis and immunology of *M. tuberculosis* infection as the profile of disease represents the full spectrum of human *M. tuberculosis* infection (Capuano *et al.*, 2003). BCG vaccination has been shown to protect cynomolgus macaques challenged intratracheally with 3,000 cfu *M. tuberculosis*, resulting in >2-log reduction of the bacterial loads and diminished lung pathology (Langermans *et al.*, 2001).

It has been proposed that the cynomolgus model of TB more closely resembles human disease than the rhesus model (Walsh *et al.*, 1996). First, cynomolgus monkeys inoculated with moderate or low doses of *M. tuberculosis* develop a chronic, slowly progressive form of TB, similar to that of adult humans. Secondly, a significant proportion of cynomolgus macaques administered a low dose of *M. tuberculosis* are able to contain the infection in a subclinical state similar to humans (Walsh *et al.*, 1996). However, others have reported asymptomatic TB in the rhesus model (Gormus *et al.*, 2004), contradicting this argument.

Immunological reagents to determine lymphocyte subsets and cytokine levels for humans can be used for cynomolgus macaque samples. A wide range of human immunochemical assays have been screened for use with this species; however, it was noted that spurious values can be obtained with different kits for the same cytokine (Verdier *et al.*, 1995). Additionally, anti-human monoclonal antibodies have been assessed for their cross-reaction with various antigens from cynomolgus monkeys with good success allowing flow cytometric analysis to be carried out (Yoshino *et al.*, 2000). This is also identical to rhesus macaques, where human reagents cross-react allowing detailed immunological analysis to be undertaken.

1.4 IMMUNE RESPONSES IN TUBERCULOSIS

The innate response is the first line of host defence against pathogens, including *M. tuberculosis,* with the adaptive response becoming prominent after several days as antigen-specific cells have undergone clonal expansion (Chaplin, 2006). It should be noted that dissecting innate and acquired host defence mechanisms is an artificial approach, as in real life the two components are complementary and synergistic (van Crevel *et al.*, 2002).

1.4.1 Innate immunity

The innate immune systems includes: (i) the epithelial barriers and the mucociliary surfaces; (ii) soluble proteins and bioactive small molecules that can be present in biological fluids (e.g. complement) or are secreted from activated cells (e.g. cytokines); and (iii) cell surface receptors that bind molecular markers on the surfaces of microbes (Chaplin, 2006).

1.4.1.1 Cells involved

1.4.1.1.1 Alveolar macrophages

Macrophages and dendritic cells are the primary cell types involved in the innate immune response to mycobacteria. However, they also play a crucial role in the initiation of adaptive immunity (van Crevel *et al.*, 2002). Alveolar resident macrophages are the primary cell types involved in the initial encounter and uptake of *M. tuberculosis* in the lung (van Crevel *et al.*, 2002). Infection with *M. tuberculosis* induces the release of interferon (IFN)-γ by human alveolar macrophages (Fenton *et al.*, 1997), suggesting that this early release of IFN-γ may play a role in innate immunity against *M. tuberculosis*. It has been shown that IFN-γ secretion, along with tumour necrosis factor (TNF)-α, is needed to stimulate macrophages to produce interleukin (IL)-12 (Flesch *et al.*, 1995). Secreting IL-12 shortly after mycobacterial challenge established the long-term persistence of IFN-γ-secreting T cells (Lagranderie *et al.*, 2003). IL-12 plays an irreplaceable role in the initiation of Th1 responses, and the loss of its function cannot be compensated for by alternative mechanisms in the lung (Wakeham *et al.*, 1998).

Another response to intracellular infection by *M. tuberculosis* is apoptosis of alveolar macrophages (Kornfeld *et al.*, 1999). Highly attenuated mycobacterial stains (e.g. H37Ra and BCG) are significantly more potent inducers of apoptosis than virulent strains such as H37Rv, Erdman, and wild-type *M. bovis* (Kornfeld *et al.*, 1999). This apoptosis mechanism eliminates the protected intracellular environment required for bacterial replication and latency (Keane *et al.*, 1997; Kornfeld *et al.*, 1999), and this may in part explain why the more virulent strains are less apoptotic.

1.4.1.1.2 Dendritic cells

Dendritic cells and monocyte-derived macrophages also take part in the phagocytic process in the lung (Henderson *et al.*, 1997; Hope *et al.*, 2004). Dendritic cells lining the trachea encounter mycobacteria that have not reached the lung, and thus are also likely to be responsible for the ensuing immune response (Hope *et al.*, 2004). Dendritic cells have multiple roles: they participate in innate immunity by internalising bacteria at the site of infection and represent the main link between innate and adaptive immunity via trafficking from lung to lymph node (Hope *et al.*, 2004; Lanzavecchia & Sallusto, 2001).

After detecting microbial products or proinflammatory cytokines, immature dendritic cells transform into mature dendritic cells with a reduced capacity for antigen uptake but with an increased capacity for T cell stimulation (Mellman & Steinman, 2001). *M. tuberculosis*-infected dendritic cells have been shown to become activated (Henderson *et al.*, 1997) and to be superior to macrophages in stimulation of antigen-specific cytokine production by both $CD4^+$ and $CD8^+$ T cells (Serbina & Flynn, 1999). Whilst activated macrophages are capable of killing intracellular bacteria, *M. tuberculosis* within dendritic cells are not killed, suggesting that dendritic cells may serve as a reservoir for *M. tuberculosis* in tissues, including the lymph nodes and lungs (Bodnar *et al.*, 2001). It is unsure whether this mechanism benefits the host or the pathogen as a continuous supply of antigen from a living bacterium may be advantageous for the priming and maintenance of an effective immune response in the host (Bodnar *et al.*, 2001). Alternatively, it may be that *M. tuberculosis* has evolved a strategy to evade killing by the dendritic cells and uses this cell to disseminate from the lung to the lymph nodes and other organs (Bodnar *et al.*, 2001).

1.4.1.2 Effector functions of the innate immune system

The major effector functions of mononuclear phagocytes include: generation of reactive oxygen intermediates; production of reactive nitrogen intermediates (RNI); limitation of intracellular iron availability; phagosome acidification plus phagosome-lysosome fusion; and production of defensins (Kaufmann, 1993).

RNI are formed from the amino acid L-arginine by the inducible nitric oxide synthase (iNOS) found in the cytoplasm of macrophages (Flesch & Kaufmann, 1993). *M. tuberculosis* is sensitive to nitric oxide (NO) made by iNOS, but whether enough iNOS can be induced in human macrophages to produce sufficient levels of NO remains controversial (Boom *et al.*, 2003). RNI play a more important role than reactive oxygen intermediates (ROI) in the expression of macrophage anti-mycobacterial activity (Sato *et al.*, 1998). This may be because *M. tuberculosis* resists killing by oxygen radical intermediates through enzymes such as superoxide dismutases (Boom *et al.*, 2003). Alternatively, it could be due to mycobacterial components, such as glycolipids, which are potent oxygen radical scavengers (Chan *et al.*, 1989).

In contrast to the normal course of events during which the phagocytosed material is taken to lysosomes where it is efficiently destroyed, following uptake, mycobacteria are able to block their delivery to lysosomes (Houben *et al.*, 2006). The cell wall protects the bacteria and promotes their intracellular persistence by inhibiting phagosome-lysosome fusion, keeping them sequestered away from terminal endocytic organelles (Deretic & Fratti, 1999). In so doing, these pathogens manage to circumvent immediate destruction, enabling them to establish a niche inside the macrophage where they can survive and even replicate (Houben *et al.*, 2006). Mycobacteria have evolved strategies to enhance

their survival and replication within the macrophage by preventing maturation of the phagosomes which contain them (Kornfeld *et al.*, 1999), leading to persistent infection.

Functional Toll-like receptors (TLRs) are required for phagocytosis to lead to immune activation (van Crevel *et al.*, 2002). The primary function of the TLR is to signal that microbes have breached the body's barrier defences by recognition of common structural features of microbes known as pathogen-associated molecule patterns (PAMPs) (Chaplin, 2006; Schnare *et al.*, 2006). Two TLRs (TLR2 and TLR4) have been implicated in the activation of macrophages by mycobacteria (reviewed by (Collins & Kaufmann, 2001; Schnare *et al.*, 2006)). TLRs direct the innate immune responses, and these responses in turn guide the subsequent adaptive immune reaction that is even more targeted (Wickelgren, 2006). In mice Th1 cells are stimulated by TLR2, but not by other TLRs, and directly induce IFN-γ production, cell proliferation, and cell survival without T-cell receptor (TCR) stimulation (Imanishi *et al.*, 2007).

1.4.2 Adaptive immunity

Two to three weeks after infection with *M. tuberculosis*, T-cell immunity develops, with antigen-specific T lymphocytes that arrive at the site of infection, proliferate within the early lesions or tubercles, and then activate macrophages to kill the intracellular mycobacteria (van Crevel *et al.*, 2002).

1.4.2.1 $CD4^+$ lymphocytes

$CD4^+$ T cells recognise antigens presented by MHC class II molecules. For these antigens to become presented on MHC class II molecules they need to be processed in

phagosomal or phagolysosomal compartments in professional antigen-presenting cells (van Crevel *et al.*, 2002). As *M. tuberculosis* can reside in these areas, $CD4^+$ T cells are thus extremely important in TB immunity.

Transgenic mice deficient in either MHC class II or CD4 molecules demonstrate increased susceptibility to *M. tuberculosis* compared to wild-type mice (Caruso *et al.*, 1999). Although IFN-γ production was reduced at 2 weeks post-infection, by 4 weeks the $CD8^+$ T cells were producing as much IFN-γ as wild-type controls (Caruso *et al.*, 1999). However, these data suggest that early production of IFN-γ by $CD4^+$ T cells is essential to control *M. tuberculosis* infection, and that IFN-γ production by other cells cannot substitute for the $CD4^+$ T cell contribution. Other investigators have also reported that in mice the majority of IFN-γ is produced by $CD4^+$ T cells early in infection (Serbina & Flynn, 1999).

Antibody-induced depletion of $CD4^+$ cells in mice latently infected with *M. tuberculosis* causes reactivation of disease, despite the continuous presence of IFN-γ and iNOS (Scanga *et al.*, 2000). These results suggest that $CD4^+$ cells may have an additional role, independent of IFN-γ production, in preventing the reactivation of tuberculosis. Some have suggested that this role may involve $CD4^+$ T cells having a cytolytic function (Tan *et al.*, 1997), which would lyse *M. tuberculosis*-infected cells (Canaday *et al.*, 2001).

Further proof that $CD4^+$ T cells are essential players in TB immunity is illustrated by increased incidence of tuberculosis in people infected with HIV (Barnes & Modlin, 1996), as an increased susceptibility to TB is seen in these individuals with low $CD4^+$ T cell counts (Havlir & Barnes, 1999). This observation has also been confirmed in SIV-infected macaques, where the reactivation of SIV-related tuberculosis-like disease

coincided with a severe depletion of $CD4^+$ T cells (Shen *et al.*, 2004). However, in HIV-infected patients, there was no correlation between the $CD4^+$ T cell count and *M. tuberculosis*-induced IFN-γ production, suggesting that $CD8^+$ T cells were the most likely source of IFN-γ production in these patients (Sodhi *et al.*, 1997).

1.4.2.2 $CD8^+$ lymphocytes

$CD8^+$ T cells recognise mycobacterial proteins presented on MHC class I molecules, which are expressed on all nucleated cells (van Crevel *et al.*, 2002). Using mice which lacked MHC class I and class I-like molecules (β2m-gene disrupted), functional $CD8^+$ T cells were found to be a necessary component of a protective immune response to *M. tuberculosis* (Flynn *et al.*, 1992). β2m gene-disrupted mice succumbed to *M. tuberculosis* infection quicker than controls, indicating that $CD4^+$ T cells alone were not sufficient to control *M. tuberculosis* infection (Flynn *et al.*, 1992). When these results were further investigated, it was found that not only $CD8^+$ T-cells were affected in β2m-gene disrupted mice, but also CD1d-restricted T cells, as well as other class I-restricted cell types which were not determined (Sousa *et al.*, 2000).

The kinetics of $CD8^+$ T cells in the lung have been shown to be identical to those of $CD4^+$ T cells (Feng *et al.*, 1999), being present during both early and late stages of *M. tuberculosis* infection. In mice, *M. tuberculosis*-specific $CD8^+$ T cells have been detected as early as 3 weeks post-infection and have been shown to be recruited to the lung (Kamath *et al.*, 2004). Using CD8-knockout mice, increased bacterial load in the lungs was a relatively late event, suggesting that $CD8^+$ T cells may be more important in controlling the chronic phase of the disease (Orme & Cooper, 1999). This was also reported in another mouse study, where depletion of $CD8^+$ cells during the latent phase

of infection resulted in a 10-fold increase in lung bacterial load (van Pinxteren *et al.*, 2000a).

$CD8^+$ T cells are able to lyse antigen-presenting cells, and have been shown to preferentially recognise heavily infected cells (Lewinsohn *et al.*, 2003). The central role of $CD8^+$ T cells may be that of surveillance, where they recognise cells in which the containment of *M. tuberculosis* is no longer effective (Lewinsohn *et al.*, 2003). This may be due to a high bacterial load leading to increased entry of mycobacterial antigens into the cytosol, or with heavy infection the early endosome may become disrupted allowing entry of antigens into the cytosol.

Human $CD8^+$ T cells have been demonstrated to directly kill intracellular mycobacteria via a granule-associated protein, granulysin (Stenger *et al.*, 1998). Using transgenic mice, $CD8^+$ T-cells only functioned if they produced IFN-γ, suggesting that they may exert their effects through cytokine-mediated macrophage activation rather than through a cytotoxic mechanism (Tascon *et al.*, 1998). However, mice do not have a granulysin homologue, so data from murine studies detailing the role of $CD8^+$ T cells should take into account the absence of this key mechanism by which $CD8^+$ T cells participate in anti-tuberculosis immunity (Lazarevic & Flynn, 2002).

$CD8^+$ T cells may play a role in protective immunity, as *M. tuberculosis*-specific $CD8^+$ cytotoxic T-lymphocyte (CTL) responses have been detected in asymptomatic contacts over a period of almost 2 years (Pathan *et al.*, 2000). However, this study only reported CTL responses in two people. *M. tuberculosis*-specific $CD8^+$ responses were also found in 4 out of 39 infected individuals, but this may be an underestimation of $CD8^+$ responses due to: (i) epitopes being restricted through only certain HLA class I alleles; (ii) *M. tuberculosis* secreting many other antigens; and (iii) $CD8^+$ cells having been at the site of

infection and not circulating (Lalvani *et al.*, 1998). Therefore, the other *M. tuberculosis*-infected individuals may have had $CD8^+$ responses that were not detected using the methods employed in the report. As mycobacteria-specific $CD8^+$ T cells were detected in patients without any symptoms of TB disease, it was hypothesised that they contributed to protection against *M. tuberculosis* growth.

In human *M. tuberculosis* infection, reactive $CD8^+$ T cells produce IFN-γ in response to stimulation with *M. tuberculosis*-infected target cells (Lewinsohn *et al.*, 1998). However, in infected mice there is little IFN-γ production by activated $CD8^+$ T cells in the lungs, so it was proposed that their role was in cytotoxicity (Serbina & Flynn, 1999). It has been shown that $CD8^+$ T cells use different effector functions during acute and chronic phases of the immune response, where $CD8^+$ T cells produce negligible amounts of IFN-γ early in infection, but switch to cytokine production during the chronic stage of infection (Lazarevic *et al.*, 2005a). Therefore, the function of the $CD8^+$ T cells is dependent on the stage of *M. tuberculosis* infection.

Using macrophages infected with *M. tuberculosis* it was shown that by adding $CD8^+$ T cells antimycobacterial activity was observed, but this did not correlate with macrophage lysis or the induction of apoptosis of infected cells (Brookes *et al.*, 2003). Instead, as cell contact was required, it was identified that $CD8^+$ T cells turned on an activation signal, subsequently found to be through receptors that included CD95 (Fas) (Brookes *et al.*, 2003).

Through their many functions, including cytotoxicity, cytokine production and macrophage activation, it is evident that $CD8^+$ T cells make an important contribution to the immune defence against *M. tuberculosis*.

1.4.2.3 Other T cell subsets

$CD4^+$ and $CD8^+$ T cells have T cell receptors (TCRs) composed of two glycoprotein chains called α- and β- TCR chains, and are thus referred to as αβ T cells. However, in γδ T cells, the TCR is made up of one γ-chain and one δ-chain. γδ T cells react to phosphoantigen components of mycobacteria in a non-MHC restricted manner (Cairo *et al.*, 2007). γδ T cells might help to control mycobacterial infection in the period between innate and classical adaptive immunity (Dieli *et al.*, 2003).

γδ T cells in the lungs of BCG-infected mice secrete IFN-γ (Dieli *et al.*, 2003) and have been shown to be more efficient producers of IFN-γ than $CD4^+$ T cells after stimulation with mycobacteria-primed autologous monocytes (Tsukaguchi *et al.*, 1995). IFN-γ is already secreted by mycobacterium-reactive γδ cells before αβ T cells come into play (Vanham *et al.*, 1997), due to the peak of γδ T cell expansion being seen at 7 days post-infection compared to 30 days for the peak of αβ T cell expansion (Dieli *et al.*, 2003). Unlike $CD4^+$ T cells though, γδ T cells do not respond to culture filtrate antigens of PPD (Tsukaguchi *et al.*, 1995). The recognition of nonpeptide antigens is conserved in non-human primates, including rhesus macaques (Cairo *et al.*, 2007; Wang *et al.*, 2003) and cynomolgus macaques (Cairo *et al.*, 2007), suggesting that non-human primates can provide an animal model for human γδ T cell antigen-specific responses.

γδ T cells have been demonstrated to be cytotoxic against BCG-infected peritoneal macrophages (Dieli *et al.*, 2003). However, it has been shown that although γδ T cells are cytotoxic to BCG-infected monocytes, they are not cytotoxic against BCG-infected dendritic cells (Martino *et al.*, 2007).

CD1-restricted T cells, like γδ T cells, do not react with mycobacterial proteins (van Crevel *et al.*, 2002). Instead, these T cells present mycolic acids, lipoarabinomanan and other components of the mycobacterial cell wall bound to CD1 on antigen-presenting cells (Kaufmann, 2003; Porcelli & Modlin, 1999). The CD1 molecules are divided into two groups – Group I (CD1a,b,c) and Group II (CD1d). Humans possess both groups, whereas in rabbits and mice only group II CD1d is present (Collins & Kaufmann, 2001). Guinea pigs, like humans, express group I CD1 molecules (Kaufmann, 2001), so experiments concerned with the role of CD1-restricted antigens can, therefore, take advantage of the guinea pig as an experimental model (Kaufmann, 2003). Macaques also have conserved group I (Morita *et al.*, 2008) and group II CD1 molecules (Saito *et al.*, 2005).

1.4.3 Humoral immunity

Until recently, humoral immunity has been somewhat neglected in TB research due to the mycobacteria residing within cells. However, it is important to note that intracellular pathogens can be found in the extracellular space during their life cycle, either before entering into host cells or after cell death, and can then be easily reached by antibodies (Glatman-Freedman, 2006). Antibody-producing B cells have been shown to modulate the host response and are involved in optimal containment of the tubercle bacillus (Maglione *et al.*, 2007). The importance of $CD4^+$ T cells for adequate B cell responses is known; understanding the effects of $CD4^+$ T cells on these cells in TB awaits a clearer picture of the role of B cells in this infection (Flynn & Chan, 2001a).

Evidence of antibody responses in TB have been observed. Patients with active TB had higher mycobacteria-specific IgG antibody responses than patients without TB (Raqib *et al.*, 2003). Children with disseminated TB had significantly lower IgG levels to LAM as

compared to controls with localised disease (Costello *et al.*, 1992). It has also been demonstrated that the ESAT-6 antigen of *M. tuberculosis* and *M. bovis* contains multiple B-cell epitopes, as assessed in infected macaques (Kanaujia *et al.*, 2004).

When high dose immunoglobulin (Ig) was given to *M. tuberculosis* infected mice a reduction in bacterial loads was observed (Roy *et al.*, 2005). However, no inhibitory effect was seen *in vitro* using *M. tuberculosis* infected macrophages or when using nude mice, indicating that T cells were required as part of the process (Roy *et al.*, 2005).

The presence of antibodies at the site of infection, prior to the entry of *M. tuberculosis* into cells, may be of particular importance with regard to the fate of infection (Glatman-Freedman, 2003). Because *M. tuberculosis* bacilli enter the lungs in small numbers, specific IgA that is present at pulmonary sites could help to attack invading pathogens on first encounter (Kaufmann, 2006a). With the help of complement, such IgA could directly damage *M. tuberculosis* bacilli and may form part of a third generation of TB vaccines (Kaufmann, 2006a). The intranasal administration of an IgA monoclonal antibody in mice has been shown to confer some protection against aerosol or intranasal *M. tuberculosis* challenge (Williams *et al.*, 2004), which was further improved by the co-administration of IFN-γ (Reljic *et al.*, 2006).

1.4.4 Granuloma formation

If *M. tuberculosis* survives its initial encounter with the alveolar macrophage a granulomatous response may develop. As the granuloma develops, monocytes enter from the blood in large numbers via an adjacent arteriole, giving rise to a characteristic perivascular cuffing, with some differentiating into epithelioid cells (Orme & Cooper, 1999). As granuloma formation continues, localised collections of epithelioid giant cells,

macrophages, newly recruited monocytes, and lymphocytes develop (Kornfeld *et al.*, 1999; Orme & Cooper, 1999). Within granulomas, the intricate interplay between T cells, cytokines and mononuclear phagocytes succeeds in containing pathogenic mycobacteria, but may fail to fully eradicate them (Kaufmann & Andersen, 1998).

The granuloma is highly successful in containing *M. tuberculosis* in distinct foci and in preventing dissemination. However, in some individuals the granuloma may develop into necrotic lesions or cavitate, both of which cause damage to the lung (figures 1.2 and 1.3) (Kaufmann, 1993). In the latter example, the caseous cavity provides an excellent habitat for extracellular *M. tuberculosis* to increase, which then leads to further lung damage and dissemination by the haematogenic or aerogenic routes (Kaufmann, 1993).

For granuloma formation cell recruitment is a highly orchestrated event regulated largely by cytokines produced by T lymphocytes (Schaible *et al.*, 1999).

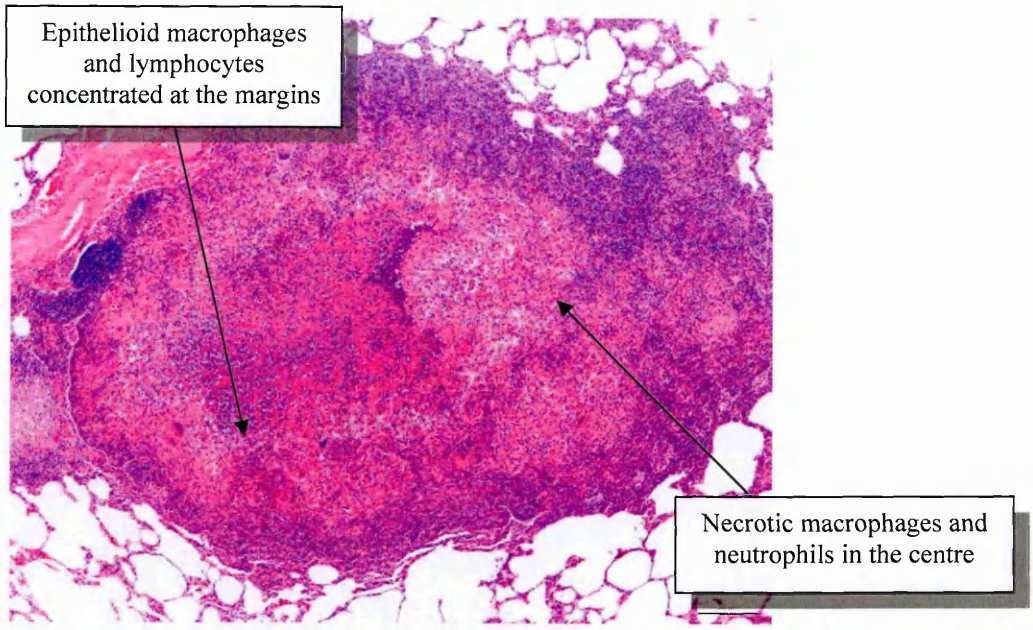


Figure 1.2: Example of a "neutrophilic" lung granuloma.

(Picture taken from a rhesus macaque after *M. tuberculosis* infection. Interpretation provided by CEPR Histology department).

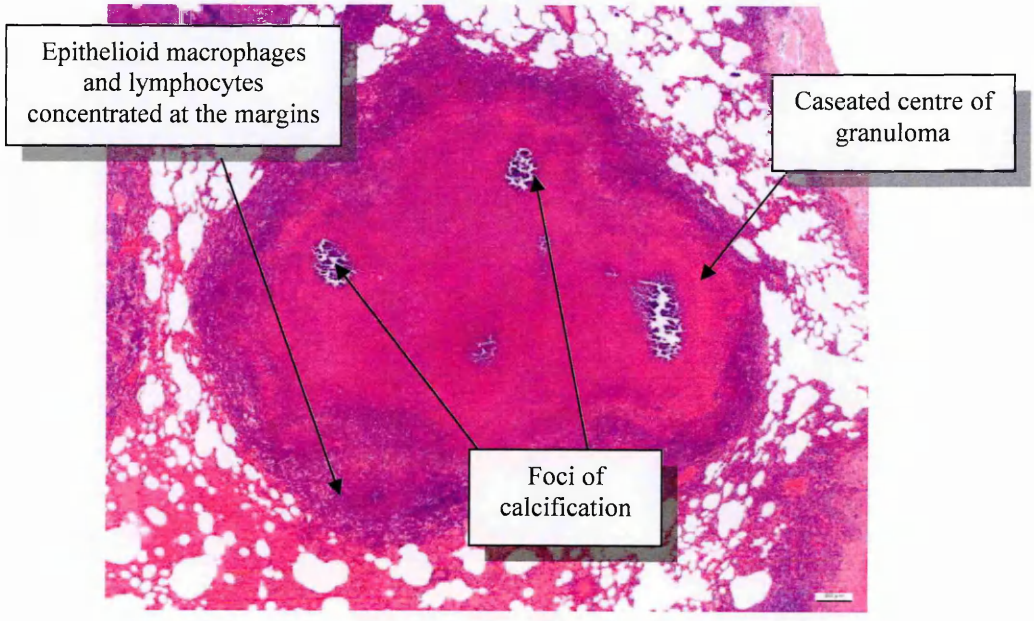


Figure 1.3: Example of a "caseated" lung granuloma.

(Picture taken from a cynomolgus macaque after *M. tuberculosis* infection. Interpretation provided by CEPR Histology department).

1.4.5 Involvement of cytokines

Cytokines are small soluble proteins secreted by one cell that can alter the behaviour or properties of the cell itself or another cell. Most cytokines have a multitude of functions, dependent on where they are secreted and their target cell.

1.4.5.1 Cytokine classification

In 1986, Mossman *et al.* showed that cloned lines of murine $CD4^+$ T cells were classified into two groups: T helper (Th)1 and Th2, based on the cytokine profile they produced (Mosmann *et al.*, 1986). Th1 cells were defined by their production of interleukin (IL)-2, IFN-γ and TNF-β; Th2 cells were defined by their production of IL-4, IL-5, IL-6, IL-10 and IL-13. Both cell types produced IL-3, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF). In 1995, this viewpoint was challenged as being too simplistic, where instead it was proposed different combinations of cytokines could be secreted with no distinct classification of cell types (Kelso, 1995). The following year, Mosmann *et al.* published work in the same journal defining further subsets of T cells (cytotoxic T-cells (Tc)-1 and Tc-2) and re-iterated that the Th1-Th2 dichotomy remained an important functional division in the immune response (Mosmann & Sad, 1996). Recently, new Th subsets are being reported including IL-17-secreting Th17 cells (Steinman, 2007).

1.4.5.2 Major cytokines involved in tuberculosis

Some of the most studied cytokines in TB and their effects are listed in table 1.3. However, cytokines often work in synergy with one another. For example, when combined, IFN-γ, TNF-α and calcitriol (1,25 (OH_2), vitamin D_3) induced a significant amount of intra-monocyte killing of *M. tuberculosis* (Denis, 1991b; Rook *et al.*, 1987). The role of some of the major cytokines in TB disease are briefly described, with the exception of IFN-γ that is covered separately in section 1.4.5.3.

1.4.5.2.1 IL-1β

The main action of IL-1β is the attraction of phagocytes (Sharma & Bose, 2001), although being an endogenous pyrogen this cytokine may also contribute to raised body temperature seen in TB patients (Dinarello, 1984).

1.4.5.2.2 IL-2

IL-2 is a T-cell growth factor that enables the population of antigen-specific T cells to expand (Sharma & Bose, 2001). In TB patients it has been shown that low-dose IL-2 therapy reduces bacterial load (Johnson *et al.*, 1995) suggesting that this cytokine was in sub-optimal concentrations in these individuals.

1.4.5.2.3 IL-6

IL-6 is critical to resistance against tuberculosis, potentially by inducing the inflammatory response and promoting IFN-γ production (Ladel *et al.*, 1997; Saunders *et al.*, 2000). However, other investigators have shown that IL-6 inhibits macrophage responses to IFN-γ (Nagabhushanam *et al.*, 2003) and T cell responses (VanHeyningen *et al.*, 1997) including suppressing synthesis of IL-1β and TNF-α (Schindler *et al.*, 1990). IL-6 is also involved in enhancing B-cell growth and differentiation (Sharma & Bose, 2001), so may contribute to the antibody response in TB. These differing effects are likely due to which cell type the cytokine interacts with, the concentration of IL-6, and the presence of other cytokines which might enhance or augment IL-6 function.

1.4.5.2.4 IL-10

IL-10 has been shown *in vitro* to be capable of suppressing IFN-γ production from alveolar macrophages stimulated with *M. tuberculosis* H37Ra (Fenton & Vermeulen, 1996). Additionally, an increase in IL-10 was associated with decreased IFN-γ production by dendritic cells stimulated with *M. tuberculosis* cell extract (Latchumanan *et al.*, 2005). It has been suggested that by hindering the antimycobacterial action of IFN-γ, IL-10 helps maintain mycobacterial infection (Murray *et al.*, 1997). However, using multidrug-resistant *M. tuberculosis* strains, others have found that IL-10 does not have a dominant role in defective IFN-γ production in patients (Lee *et al.*, 2002).

1.4.5.2.5 IL-12

IL-12 contributes to the development of precursor T cells into Th1 cells (Trinchieri, 1995), which are required to generate a successful immune response against *M. tuberculosis*. This cytokine is essential for control of mycobacterial infection, as shown by increased levels of infection with weakly pathogenic mycobacteria in patients genetically deprived of IL-12 immunity (Altare *et al.*, 1998; de Jong *et al.*, 1998) and in IL-12 knockout mice (Cooper *et al.*, 1997). However, its effects are also immunoregulatory, limiting pathology associated with aerosol *M. tuberculosis* infection in mice (Nolt & Flynn, 2004) and regulating IFN-γ production (Magram *et al.*, 1996).

1.4.5.2.6 TNF-α

Studies in TNF-α-deficient mice have shown that this cytokine is important in the aggregation of leukocytes into functional granulomas capable of controlling virulent mycobacterial infection (Roach *et al.*, 2002). TNF-α-deficient mice also have diminished recruitment and activation of T cells and macrophages into the lung (Botha & Ryffel, 2003). This may be due to changes in chemokine production leading to a cascade of inappropriate cellular communication, impaired cell migration within the lung, loss of cell-mediated immune response, and substantial lung pathology (Aronson *et al.*, 2004). Without TNF-α, granulomas are still formed in equal numbers to control mice, but necrosis is observed as is neutrophilic infiltration of the alveoli (Bean *et al.*, 1999; Flynn *et al.*, 1995).

Excessive production of TNF-α may lead to necrosis in the lung, and if released into the systemic circulation may contribute to symptoms including fever (Sharma & Bose,

2001). TNF-α also immunoregulates inflammatory cytokines, to prevent overproduction that could result in immunopathology (Mohan *et al.*, 2001; Zganiacz *et al.*, 2004). Therefore, TNF-α exhibits both advantageous and detrimental effects in TB disease.

Cytokine	Function	Reference
IFN-γ	• Activates macrophages to kill intracellular mycobacteria. • Involved in production of reactive nitrogen intermediates. • Increases MHC and co-stimulatory molecule expression. • Plays role in cell death. • Down-regulates the macrophage mannose receptor.	(Bonecini-Almeida *et al.*, 1998) (Flynn *et al.*, 1993) (Boom *et al.*, 2003) (Liu & Janeway, 1990) (Fenton & Vermeulen, 1996)
TNF-α	• Recruitment and activation of T cells and macrophages into the lung. • Essential for granuloma formation. • Affects expression of chemokines by macrophages *in vitro*. • Immunoregulates type 1 immune responses. • Production of reactive oxygen species by activating neutrophils.	(Botha & Ryffel, 2003) (Bean *et al.*, 1999; Botha & Ryffel, 2003; Roach *et al.*, 2002) (Algood *et al.*, 2004) (Zganiacz *et al.*, 2004) (McDermott, 2001)
IL-1β	• Proinflammatory cytokine. • Required for granuloma formation.	(van Crevel *et al.*, 2002) (Juffermans *et al.*, 2000)
IL-4	• Suppression of IFN-γ production. • Required for the generation of Th2-derived cytokines. • May play a protective role in controlling mycobacterial infection.	(Powrie & Coffman, 1993) (Kopf *et al.*, 1993) (Sugawara *et al.*, 2000)
IL-6	• Induction of T-cell mediated resistance to *M. tuberculosis* and *M. avium*.	(Appelberg *et al.*, 1994; Ladel *et al.*, 1997)
IL-10	• Antagonises the proinflammatory cytokines by downregulation of IFN-γ, TNF-α and IL-12.	(Gong *et al.*, 1996)
IL-12	• Initiation of Th1 responses. • Crucial for protection against *M. tuberculosis* by inducing IFN-γ.	(Wakeham *et al.*, 1998) (Cooper *et al.*, 1997)
IL-15	• Stimulates T cell and natural killer cell proliferation and activation. • Causes selective stimulation of memory-phenotype $CD8^+$ cells in vivo.	(Doherty *et al.*, 1996; Liu *et al.*, 2000) (Zhang *et al.*, 1998)
IL-17	• Enhances IFN-γ secretion and granuloma formation. • Involved in lymphocyte migration to the lungs.	(Umemura *et al.*, 2007) (Kolls & Linden, 2004)
IL-18	• Contributes to type 1 cytokine responses, including inducing IFN-γ.	(Hunter & Reiner, 2000; Sugawara *et al.*, 1999; van Crevel *et al.*, 2002; Vankayalapati *et al.*, 2000)

Table 1.3: Cytokines involved in immunity against *M. tuberculosis* infection and their roles.

1.4.5.3 Interferon-γ in tuberculosis

One of the key roles of IFN-γ is to aid with the activatation of macrophages to kill intracellular mycobacteria along with lymphocyte cells (Bonecini-Almeida *et al.*, 1998). Without the addition of IFN-γ, macrophages were unable to reduce the bacterial load of *M. tuberculosis* in an *in vitro* system. In IFN-γ gene-knockout mice, animals failed to produce reactive nitrogen intermediates and were unable to restrict the growth of *M. tuberculosis* (Flynn *et al.*, 1993). This has been confirmed using *in vitro* macrophage assays, where IFN-γ was shown to be capable of inducing both NO_2^- (a reactive nitrate intermediate) and H_2O_2 (a reactive oxygen intermediate) release (Ding *et al.*, 1988; Sato *et al.*, 1998).

Mice in which the IFN-γ gene has been disrupted are unable to contain or control a normally sub-lethal dose of *M. tuberculosis*, delivered either intravenously or aerogenically (Cooper *et al.*, 1993). This indicates that IFN-γ plays a pivotal role in protective cellular immunity to TB infection. Patients with genetic alterations in the IFN-γ receptor have also been shown to have increased susceptibility to *M. tuberculosis* infection (Cooke *et al.*, 2006; Newport *et al.*, 1996; Sallakci *et al.*, 2007). Additionally, patients with IL-12 receptor deficiencies are also at increased risk of infection (Altare *et al.*, 1998; Ottenhoff *et al.*, 1998; Ottenhoff *et al.*, 2005). This could be due to IL-12 playing an essential role in regulating IFN-γ production, as demonstrated in mice lacking IL-12 (Magram *et al.*, 1996).

IFN-γ alone does not appear to allow human macrophages/monocytes to have antituberculous properties (Rook *et al.*, 1986). In some circumstances, IFN-γ may even promote growth of intramacrophage *M. tuberculosis* (Douvas *et al.*, 1985). It may be that

in man, protection against *M. tuberculosis* depends on the appropriate exposure to immunologically active vitamins, such as vitamin D, or an interaction between vitamins, cytokines and/or primed cells (Bonecini-Almeida *et al.*, 1998; Crowle, 1990).

Mice with a targeted disruption of the IFN-γ gene have reduced expression of macrophage MHC class II antigens (Dalton *et al.*, 1993). Thus, IFN-γ is a critical regulator of antigen-presenting cell function by increasing MHC and co-stimulatory molecule expression (Boom *et al.*, 2003). This latter activity is essential for optimal T cell responses to *M. tuberculosis*.

Other functions of IFN-γ include: being required in order for $CD8^+$ T cells to exert their anti-mycobacterial effect (Tascon *et al.*, 1998); playing a critical role in cell death, which may eliminate activated effector T cells (Liu & Janeway, 1990); and down-regulating the macrophage mannose receptor, to which *M. tuberculosis* can bind in order to gain entry into macrophages (Fenton & Vermeulen, 1996). IFN-γ also has an important role in the stimulation of IgG2a secretion and in the suppression of IgG1 production (Finkelman *et al.*, 1988). However, this may be more relevant in situations where humoral immunity takes precedent.

It has been shown that during *M. tuberculosis* infection, IFN-γ production may be sub-optimal. Reduced IFN-γ production by PBMCs stimulated with heat-killed *M. tuberculosis* was observed in patients with severe disease as determined by chest radiograph and clinical presentation (Sahiratmadja *et al.*, 2007b; Sodhi *et al.*, 1997). In naïve mice given a lethal dose of *M. tuberculosis*, infusion of IFN-γ resulted in increased survival (Denis, 1991a). This observation has been transferred to humans, where some patients with mycobacterial infection respond to administration of recombinant IFN-γ

(Condos *et al.*, 1997), suggesting that there could be a relative deficiency of this cytokine in some individuals.

It is apparent that production of IFN-γ is critical for the control of *M. tuberculosis* infection, whether produced early in infection as a by-product of the activation of immune defence mechanisms, or by antigen-specific T cells following the induction of specific immunity (Collins & Kaufmann, 2001). The release of IFN-γ from $CD4^+$ and $CD8^+$ T cells remains the primary assay used to determine the immunogenicity of any new TB vaccine (Fletcher & McShane, 2006). Studies of experimental vaccines in the mouse model of TB support IFN-γ as a relevant marker for the induction of a protective immune response (Agger & Andersen, 2001). The relevance of IFN-γ as a marker for generating adequate protective immunity has been confirmed at the Statens Serum Institut (SSI) where all of the subunit vaccines tested so far showing levels of protection equivalent to BCG have given strong IFN-γ responses (Agger & Andersen, 2001). However, others have shown that increasing IFN-γ responses does not always result in increased protection against *M. tuberculosis* infection (Leal *et al.*, 2001).

1.5 ASSAYS TO MEASURE IFN-γ

As IFN-γ is shown to be such an important cytokine in TB disease, its production often requires evaluation. Two measures of IFN-γ are generally used in order to determine the kinetics of IFN-γ secretion after infection with, or vaccination against, *M. tuberculosis*: the frequency of cells capable of secreting IFN-γ and the amount of IFN-γ secreted.

1.5.1 IFN-γ ELISPOT assay

The frequency of IFN-γ-secreting cells can be assessed by an enzyme-linked immunosorbent spot (ELISPOT) assay. This technique is a modified version of the enzyme-linked immunosorbent assay (ELISA). However, the ELISPOT assay relies on capturing the substrate's colour reaction product on a solid surface as the 'footprint' of the cytokines secreted by individual cells (Cox *et al.*, 2006).

This assay is highly sensitive due to measuring cytokine production of single cells. This is in part due to the fact that the product is rapidly captured around the secreting cell before it is either diluted in the supernatant, captured by receptors of adjacent cells, or degraded. Some investigators have reported frequencies from 50 IFN-γ-producing cells/10^6 PBMC (Russell *et al.*, 2003) to less than 4 per 10^6 PBMC (Helms *et al.*, 2000). The level of detection is 10 to 200 times more sensitive than ELISA performed on culture supernatants (Tanguay & Killion, 1994). Due to the nature of the assay it is unlikely that the real frequencies of antigen-specific cells are overestimated. This may be partly due to the inability of all cells with the right specificity to produce IFN-γ and several clustered responsive cells could produce a single footprint (Mwau *et al.*, 2002).

Due to cells being restimulated with defined antigens the ELISPOT primarily measures the relative magnitude of the antigen-specific recall response, which may not reflect the responses in the subject at the time the sample was taken (Doherty *et al.*, 2005). The ELISPOT assay has been used in multicentre settings to reliably detect antigen-specific T cells induced by various vaccines, such as those to prevent cancer (Scheibenbogen *et al.*, 2000).

1.5.2 Whole blood IFN-γ ELISA assay

The IFN-γ ELISA assay measures the concentration of cytokine secreted from cells after stimulation with mycobacterial antigen.

The ELISA assay can use diluted whole blood as this mimics the natural environment with the *in vivo* cellular and humoral interactions as well as allowing modulation of cytokine production by endogenous or exogenous factors (De Groote *et al.*, 1992). Furthermore, the alternative to using whole blood is to use isolated PBMC. As polymorphonuclear cells represent about 60% of leukocytes, and monocyte concentrations are substantially reduced in PBMC preparations (e.g., due to attachment of monocytes on different structures used during the separation process), these modified cell ratios in PBMC may affect qualitative and quantitative production of cytokines (De Groote *et al.*, 1992). It has been reported that monocytes (Weir *et al.*, 2003) and macrophages (Wang *et al.*, 1999) are significant cytokine producers in mycobacterial infections, hence supporting the use of whole blood. When diluted whole blood has been compared with PBMC samples, it was found that there were significant differences in IFN-γ levels after stimulation with phytohaemagglutinin (PHA) (Silberer *et al.*, 2008). In another study, lipopolysaccharide (LPS) induced more IL-1β and IL-6 in whole blood

compared to PBMC (Schindler *et al.*, 1990). The use of whole blood makes this assay easy to use in field studies, as processing samples for lymphocyte isolation is not required, thus making the assay more practically feasible. However, for some analyses (e.g. ELISPOT) whole blood cannot be used due to background interference by blood components (e.g. erythrocytes).

In human TB studies, diluted whole blood has been incubated for 6 days in the presence of mycobacterial antigen (Black *et al.*, 2001; Black *et al.*, 2003; Dockrell *et al.*, 2000). Results from one of these studies have shown that the 6-day IFN-γ response to *M. tuberculosis* PPD has a strong association with induration measured by skin testing to the same antigen (Dockrell *et al.*, 2000). When overnight assays were compared with 6 day stimulated lymphocytes it was observed that the longer period of stimulation was more sensitive for the detection of latent TB (Leyten *et al.*, 2007). Others have also found that when comparing overnight and 5 day PBMC incubation periods with varicella-zoster antigen, *Candida albicans* antigen or hepatitis B surface antigen, natural killer (NK) cells account for most of the IFN-γ secreting cell population after overnight stimulation, whereas the T-cell IFN-γ contribution increases with the 5 day incubation (Desombere *et al.*, 2005). The investigators reported that the NK:T cell ratio of IFN-γ secreting cells went from <0.5 to >1.5 (Desombere *et al.*, 2005).

1.5.3 Luminex assay

The luminex assay can be used where a range of cytokines are to be measured. This technology uses bead-based assays capable of measuring multiple analytes in a single well. Beads specific for each cytokine can be distinguished by incorporation of a unique ratio of dyes which can be distinguished using lasers in the luminex analyser. The beads

are coated with specific anti-cytokine antibody, and captured cytokine is quantified using a fluorescence detection system.

Ready-to-use beads are available for a number of cytokines, but otherwise multiplex assays can be developed in-house if a suitable antibody pair exists (de Jager *et al.*, 2003). The assay is sensitive and accurate as each fluorescence signal is the mean of 100 measurements of a signal microsphere, and each bead represents an assay by itself (de Jager *et al.*, 2003). Luminex analysis has been used in macaques during an Ebola outbreak (Hutchinson *et al.*, 2001) using beads prepared by the investigators. However, other investigators have tested cross-reaction of non-human primate samples with commercially-available human beadsets with success for the majority of the cytokines tested (Giavedoni, 2005).

1.5.4 Intracellular cytokine staining

Intracellular cytokine staining (ICS) involves using flow cytometry to analyse cells that have synthesised cytokines. Similar to the ELISPOT assay, the readout of this assay is the frequency of cells capable of producing cytokine.

The major advantage of this assay is that in addition to staining cells with fluorescent anti-cytokine antibodies, other phenotypic characteristics of the cells can be determined in parallel (Tesfa *et al.*, 2004). This type of analysis has proved useful in showing that a significant proportion of IFN-γ-producing cells after BCG-vaccination are NK cells (Hanekom *et al.*, 2004). However, this assay in the short-term only reliably detects newly formed intracellular proteins, such as IFN-γ, which is captured in the Golgi apparatus (Hanekom *et al.*, 2004), unlike the ELISPOT assay which measures cytokine that is actually secreted by the cells.

Where both assays have been carried out simultaneously, the ELISPOT assay is more sensitive (detection limits: $1x10^{-4}$ for ELISPOT versus $1x10^{-3}$ for ICS) (Sun *et al.*, 2003). Therefore, the ELISPOT is preferable for the determination of low-level responses, whereas ICS has a greater dynamic range and also allows for phenotypic discrimination of responding cells (Karlsson *et al.*, 2003).

1.6 AIMS OF THE STUDY

1.6.1 Background to hypothesis

A high research priority for TB immunologists and vaccinologists is the identification and validation of immunological correlates of protection, and the incorporation of such assays into the routine testing of new TB vaccines in both experimental and target species (McMurray, 2001a). Understanding natural immune correlates, or biomarkers, of protection is important both in guiding vaccine design but additionally in identifying potential early markers of efficacy for vaccines (Sander & McShane, 2007). In the absence of a pre-defined immunological correlate of protection, the key question when developing a new TB vaccine is whether such significantly enhanced immune responses seen after vaccination are accompanied by an improvement in protective efficacy (Pathan *et al.*, 2007).

Induction of IFN-γ is the primary immune assay for phase I TB vaccine trials (Sander & McShane, 2007). In clinical studies, IFN-γ has been measured by *ex vivo* ELISPOT assay (McShane *et al.*, 2004), the whole-blood ELISA assay (Black *et al.*, 2002; Gorak-Stolinska *et al.*, 2006), and intracellular cytokine staining (Hanekom *et al.*, 2004). The ELISPOT assay has been shown to provide the most consistent results and is used as the

main immunological outcome in clinical trials (Hawkridge *et al.*, 2008). The whole-blood IFN-γ ELISA is considered to have an important role in the field, because of its simplicity (Sander & McShane, 2007). Whole-blood production of IFN-γ has been reported to be the best available correlate of protection (Ellner *et al.*, 2000) and indicator of protective immunity (Kaufmann, 2006a).

1.6.2 Hypothesis and objectives

The main hypothesis to be tested in this study is as follows:

"IFN-γ measurements in a non-human primate experimental model of tuberculosis provide an accurate measure of protective efficacy against *M. tuberculosis* infection."

This hypothesis will be tested by completion of the following objectives:

Objective 1: Assess differences between IFN-γ responses to BCG vaccination and *M. tuberculosis* infection in two non-human primate species: rhesus macaques and cynomolgus macaques.

Objective 2: Evaluate whether trends in production of IFN-γ after BCG vaccination in macaques can be related to those observed in humans.

Objective 3: Determine whether either the number of IFN-γ-secreting cells or the concentration of IFN-γ secreted correlates with protection against *M. tuberculosis* challenge following vaccination with BCG and a novel TB vaccine.

1.6.3 Scope of the thesis

The methods used in this thesis are detailed in **Chapter 2.**

Before vaccination and challenge work could be undertaken in non-human primates, assays needed to be performed in naïve animals to ensure that they were suitable for experimental studies. **Chapter 3** details the results for screening antigens and the background responses obtained in macaques prior to vaccination with BCG or infection with *M. tuberculosis*. From this work, a cut-off would be defined to help determine whether genuine responses occurred following vaccination or challenge.

Chapter 4 reports the measurement of IFN-γ responses after aerosol infection of macaques with *M. tuberculosis*. Comparisons between rhesus macaques and cynomolgus macaques were undertaken. In animals which succumbed to rapid disease progression, the IFN-γ measurements were analysed to determine whether they correlated with clinical progression of *M. tuberculosis* infection.

A preliminary comparison of BCG-vaccination responses in rhesus macaques and cynomolgus macaques is reported in **Chapter 5**. A larger cohort of rhesus macaques were then vaccinated with BCG, with some animals later receiving immunisation with MVA85A – a novel 'booster' TB vaccine currently undergoing evaluation in clinical trials. Data available from human clinical trials were used to compare IFN-γ secretion profiles between macaques and humans in order to assess the relevance of the animal model. Having undergone vaccination, animals were challenged with *M. tuberculosis* and IFN-γ measurements monitored for up to a year. Data were then evaluated to assess

whether IFN-γ measurements were a reliable indicator of protection against *M. tuberculosis*.

Although each chapter is discussed individually, **Chapter 6** brings together all of the results presented in the thesis and discusses their implications on the hypothesis and suggestions for further work.

2 MATERIALS AND METHODS

2.1 *IN VIVO* MACAQUE STUDIES

2.1.1 Housing and behaviour

Studies were performed on *Macaca mulatta* (rhesus macaques: mean age 45 months, range 35-65 months) and *Macaca fascicularis* (cynomolgus macaques: mean age 50 months, range 43-62 months). Macaques were housed according to the Home Office (UK) guidelines for the care and maintenance of primates. Animals were inspected daily for indications of change in behaviour. All animal work was carried out by personnel in the Biological Investigations Group and formed part of TB vaccine research in progress at the Centre for Emergency Preparedness and Response (CEPR), part of the Health Protection Agency (HPA).

2.1.2 Sample collection

For sample collection, animals were sedated with ketamine hydrochloride before removal from the cage. Freshly drawn venous blood was mixed with sodium heparin to minimise any clot formation.

2.1.3 Experimental study design

Individual experiments assessed IFN-γ responses after infection with *M. tuberculosis* and/or vaccination with BCG and/or MVA85A.

2.1.3.1 Studies assessing IFN-γ responses after *M. tuberculosis* infection

Nine rhesus macaques and twelve cynomolgus macaques were infected with *M. tuberculosis* via the aerosol route (refer to section 2.1.5). Animals from each species were divided into groups which were subsequently challenged on separate occasions over 4 months apart (table 2.1). Disease progression was monitored over a 12-week period post-challenge and samples were collected for IFN-γ analysis as shown in figure 2.1.

Animal ID			Experimental details
	Rhesus macaques	**Cynomolgus macaques**	
Challenge 1	D31	4313	Challenged with *M. tuberculosis* (follow-up period of 12 weeks)
	D53	9909013	
	D80	9909187	
Challenge 2	C54	109027	
	D19	109181	
	D60	109203	
Challenge 3	D12	1111	
	D28	1163	
	D42	2151	
Challenge 4		0111231	
		0201011	
		0201191	

Table 2.1: Identification of macaques used in *M. tuberculosis* challenge studies.

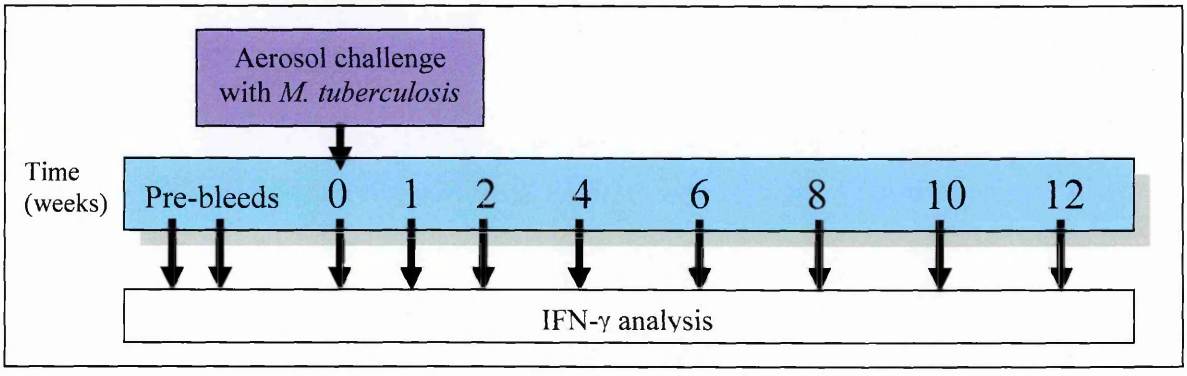


Figure 2.1: Overview of *M. tuberculosis* infection studies showing the time of sampling for IFN-γ analysis.

2.1.3.2 Study assessing longitudinal IFN-γ responses following BCG vaccination

A pilot study was performed to determine the dynamics and longevity of the IFN-γ response post-intradermal vaccination with BCG (refer to section 2.1.4.1). Two macaques of each species (rhesus and cynomolgus) were immunised with BCG (table 2.2) and samples were collected over 74 weeks as shown in figure 2.2.

Animal ID		Experimental details
1040	Rhesus macaques	Immunised with BCG (follow-up period of 74 weeks)
1252		
1007	Cynomolgus macaques	
9911021		

Table 2.2: Identification of macaques used in longitudinal BCG-vaccination study.

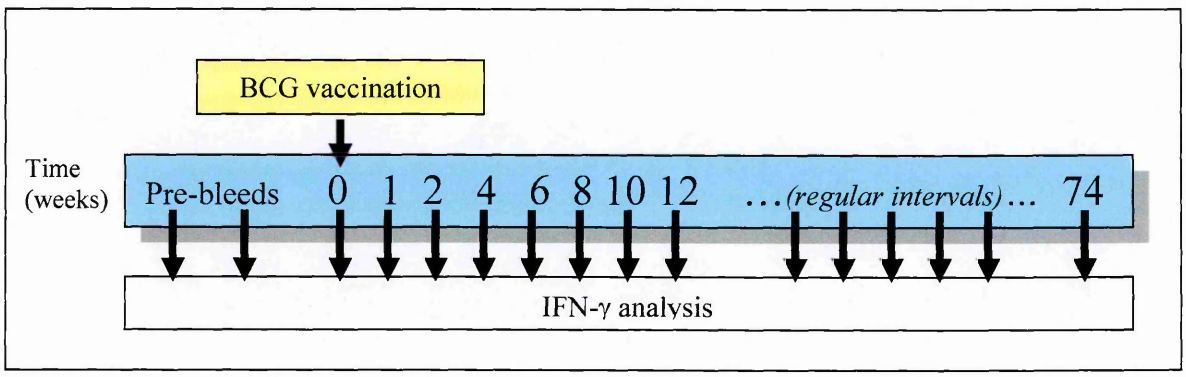


Figure 2.2: Overview of longitudinal BCG-vaccination study showing the time of sampling for IFN-γ analysis.

2.1.3.3 Study assessing IFN-γ responses after BCG and BCG/MVA85A vaccination, and following aerosol challenge with *M. tuberculosis*

Sixteen rhesus macaques were used to determine responses after vaccination with BCG, and the effects of MVA85A boosting of BCG-vaccination responses. These animals were split into three groups during the vaccination phase of the experiment: (A) BCG vaccinated; (B) BCG vaccinated with MVA85A boost; and (C) unvaccinated (table 2.3). Figure 2.3 shows a schematic overview of the experimental study; 21 weeks after the initial BCG vaccination all animals were challenged by the aerosol route with *M. tuberculosis* and followed for up to 52 weeks post-infection.

Animal ID		Immunisation/Challenge details
K80	Rhesus macaques	Group A: Immunised with BCG (for 21 weeks) and challenged with *M. tuberculosis* (follow-up period of 52 weeks)
K52		
K59		
K43		
K50		
K44		
K65		Group B: Immunised with BCG (for 12 weeks), boosted with MVA85A (for 9 weeks) and challenged with *M. tuberculosis* (follow-up period of 52 weeks)
K20		
K69		
K86		
K61		
K79		
K62		Group C: Challenged with *M. tuberculosis* (follow-up period of 52 weeks)
K54		
K32		
K47		

Table 2.3: Identification of macaques used in BCG or BCG/MVA85A vaccination studies followed by challenge with *M. tuberculosis*.

(Group C were unvaccinated control animals).

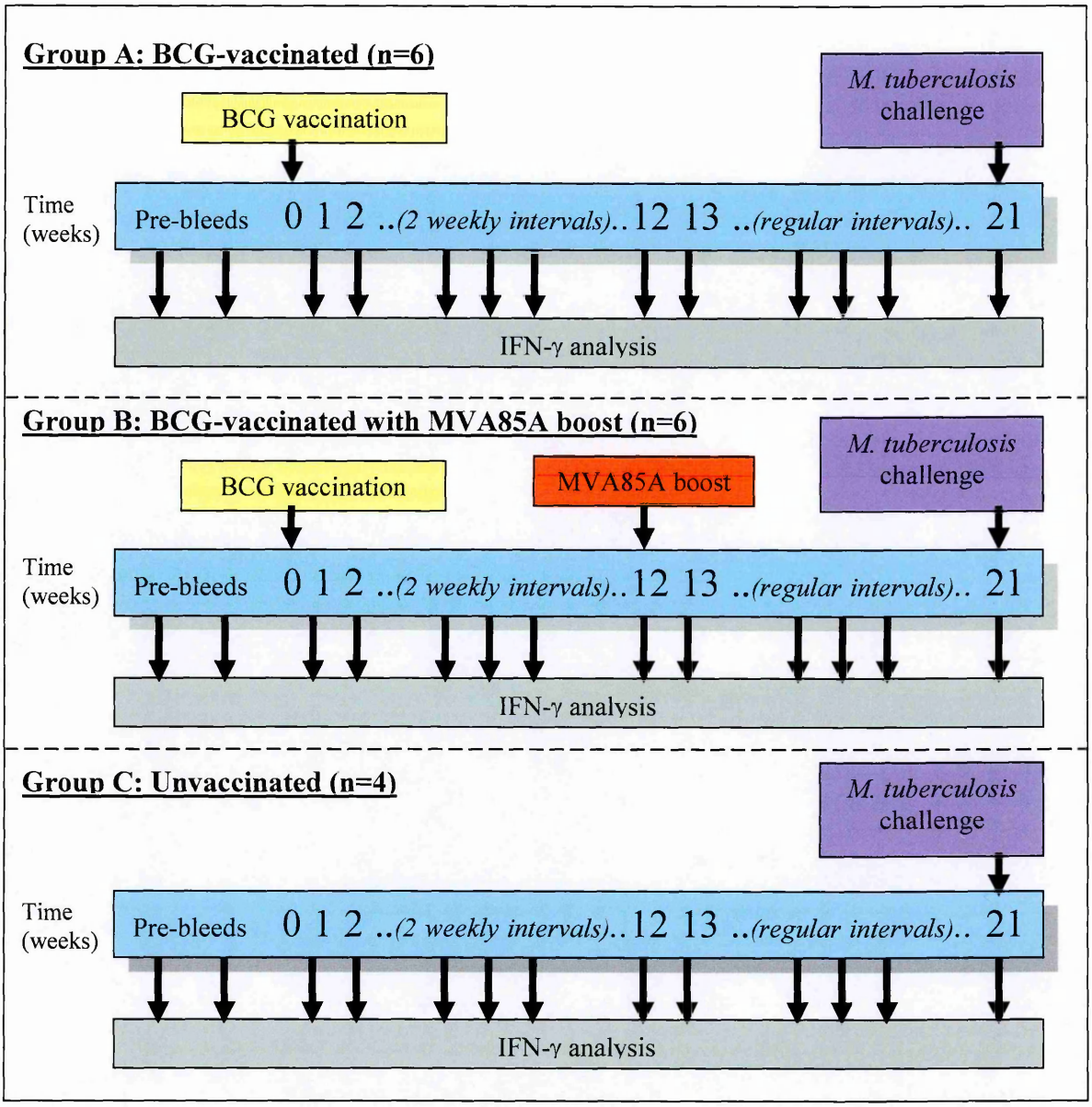


Figure 2.3: Overview of vaccination studies involving BCG and BCG with MVA85A boosting showing the sampling times for IFN-γ analysis.

(After challenge at week 21, animals were monitored for up to 52 weeks; group C were unvaccinated control animals).

2.1.4 Vaccination

2.1.4.1 BCG vaccination

Macaques were vaccinated with bacille Calmette-Guérin (BCG) (Danish strain 1331, Statens Serum Institut, Copenhagen, Denmark) via the intradermal route in the upper left arm. Vaccine vials had a concentration of 2-8x10^6 cfu/ml of BCG. Macaques in the pilot study (section 2.1.3.2) were immunised with 50 µl vaccine whereas macaques in the vaccine efficacy study (section 2.1.3.3) were immunised with 100 µl vaccine. 50 µl is equivalent to a childs dose, whereas 100 µl is the recommended dose for human adults.

2.1.4.2 MVA85A vaccination

One group of animals previously vaccinated with BCG was immunised with modified vaccinia Ankara virus expressing Ag85A vaccine (MVA85A, supplied by Helen McShane, Oxford) administered via the intradermal route in the upper right arm. The MVA85A supplied was at a concentration of 5x10^9 pfu (plaque-forming units)/ml and macaques were immunised with 100 µl vaccine, equating to a dose of 5x10^8 pfu being delivered.

2.1.5 Aerosol challenge of macaques with *M. tuberculosis*

Macaques of each species were exposed to an aerosol of *M. tuberculosis* Erdman strain (KO1, obtained from CBER/FDA) generated by a Collison nebuliser in conjunction with a Hendersen apparatus, as described by Williams *et al.* (Williams *et al.*, 2000). Nose-

only delivery was ensured by the use of a mask, and plethysmography was used to measure and control the volume of aerosol inhaled. This took into account differences in animal's size and breathing rate and was used to estimate inhaled doses. Individual doses for each of the animals are shown in appendix 1 (section 8.1).

2.1.6 Necropsy of macaques

Upon completion of experimental schedules, or when animals had reached a humane clinical endpoint (i.e. 20% weight loss from maximal weight or showing signs of malaise), animals were euthanised by the administration of an overdose of anaesthetic. Dissection of the animal and tissue collection was then performed by trained personnel experienced in non-human primate anatomy and physiology.

2.1.6.1 Pathology scoring

During the post-mortem procedure, organs in the animals were scored according to the system shown in figure 2.4. This score was developed by researchers trying to standardise protocols in assessing extent of *M. tuberculosis* disease in non-human primates (Flynn-J, Reed-S and Langermans-J. World Health Organisation: proposed recommendations for evaluation of TB vaccine efficacy in non-human primates – kindly supplied by J. Langermans). Pathology scoring was assisted by personnel who had previous experience in assessing the extent of *M. tuberculosis* infection in the guinea pig model of TB infection.

2.1.6.2 Tissue collection

At necropsy, samples were taken for immunological studies from the hilar lymph node, axillary lymph node, inguinal lymph node and spleen. These were dissected from the animal and put into a dry sterile tube.

Lung scoring system		
0	No visible lesion	
1	1 gross lesion	<10 mm diameter (in case of calcification score = 2)
2	2-5 gross lesions	<10 mm diameter
3	>6 gross lesions or 1 gross lesion	<10 mm diameter >10 mm diameter
4	>1 gross lesion	>10 mm diameter
5	Gross coalescing lesions	
Lymph node and other organs scoring system		
0	No lesion/no necrosis	
1	1 small focus	
2	Several foci/necrotic or caseous area 5x5mm or greater	
3	Extension necrosis/caseation	

Pathology score = scores from lung
(left lower lobe, left upper lobe, right lower lobe, right middle lobe and right upper lobe)
+
Scores from other tissues
(hilar lymph node, spleen, liver and other affected organs)

Figure 2.4: Diagram showing the pathology scoring of macaques at necropsy.

2.2 *EX VIVO* AND *IN VIVO* TECHNIQUES

2.2.1 Prior exposure of macaques to mycobacteria

The PRIMAGAM test kit was used to determine that none of the macaques used in the experiments had previously been exposed to environmental or pathogenic mycobacteria. This commercial diagnostic test is divided into three parts: (i) stimulation of blood; (ii) an enzyme immunoassay; and (iii) interpretation of the results. All reagents were supplied as part of the PRIMAGAM kit (Biocor Animal Health, Omaha, USA).

2.2.1.1 PRIMAGAM: stimulation of blood

Three ml heparinised blood from each animal was distributed into three wells of a 24-well plate. To the first well 2 drops of nil antigen were added, to the second well 2 drops of *M. avian* PPD, and to the third well 2 drops of *M. bovis* PPD. Blood and antigen were mixed thoroughly by swirling the plate for 1 minute and incubated at 37°C in 5% CO_2 overnight.

Plates were then spun in a plate-spinning centrifuge at 500 *g* for 10 minutes. The supernatants were then aspirated off into sterile plastic vials and stored at 2-8°C for up to a week.

2.2.1.2 PRIMAGAM: enzyme immunoassay

Pre-coated (anti-IFN-γ) strips were filled with 50 µl green diluent followed by 50 µl test sera. Each sample was tested in duplicate. 50 µl negative control solution was added to two wells, and 50 µl of positive control (recombinant IFN-γ) was added to another two wells. Samples were mixed by gently swirling the plate for 1 minute and incubated for 1 hour at room temperature.

Plates were then washed six times with 300 µl per well of wash buffer. After the last cycle the plates were dried by tapping on absorbent paper and 100 µl of diluted conjugate was added per well. Conjugate was supplied as a 100x concentrate and was diluted in green diluent solution. Plates were incubated for 1 hour at room temperature.

Plates were washed six times with 300 µl per well of wash buffer as before. Following the last wash, 100 µl diluted chromogen was added per well. This was supplied in a 100x solution, and was diluted in the supplied enzyme substrate buffer. Plates were incubated for 30 minutes at room temperature with protection from direct light achieved by wrapping the plates in foil.

To each well, 50 µl of enzyme stopping solution was added to terminate the colorimetric reaction. Absorbance values were then measured using a plate spectrophotometer at a wavelength of 450 nm. Plates were read within 5 minutes of adding enzyme stopping solution.

2.2.1.3 Interpretation of results

For the assay to be valid the absorbance values for the control samples were as follows:

- Negative control: Absorbance less than 0.150
  (replicates must not vary by more than 0.040)
- Positive control: Absorbance more than 1.000
  (replicates must not deviate by more than 15% from mean)

To determine whether animals had prior exposure to *M. tuberculosis* the absorbance would need to be 0.050 higher than those seen in nil antigen- or avian PPD-stimulated samples. The kit was also used to assess whether animals had been exposed to environmental mycobacteria by ensuring that the results in the avian PPD-stimulated samples did not exceed by 0.050 the levels in the nil antigen-stimulated samples.

2.2.2 Medium

The medium for lymphocyte isolation and assay incubation consisted of RPMI 1640 media (Sigma-Aldrich, Dorset, England) supplemented with 2mM L-glutamine (Sigma-Aldrich), 25mM HEPES buffer (Sigma-Aldrich), 100 U/ml penicillin with 100 μg/ml streptomycin (Sigma-Aldrich) and 0.05mM 2-Mercaptoethanol (Invitrogen, Paisley, England).

Media identification was distinguished by its content of fetal calf serum (FCS) (LabTech, Ringmer, England): R0 = no FCS; R2 = 2% FCS; R5 = 5% FCS; and R10 = 10% FCS.

2.2.3 Antigen preparation

2.2.3.1 Purified protein derivative

PPD produced from culture filtrates from virulent strains of *M. tuberculosis* was obtained from Statens Serum Institut (SSI, Copenhagen, Denmark) at a 1 mg/ml solution. Aliquots were stored at 2-8°C until required for use in immunological assays.

2.2.3.2 Recombinant proteins

Recombinant proteins to ESAT-6 and Ag85B (Lionex, Braunschweig, Germany) were supplied at 1.05 mg/ml and 1.02 mg/ml, respectively. Proteins were made to 1 mg/ml by diluting in sterile Phosphate Buffered Saline (PBS) (Severn Biotech Ltd, Worcestershire, England) and aliquots were frozen at -20°C until required.

2.2.3.3 Peptides

Overlapping 15mer peptides covering the sequences of Ag85A, CFP10 and ESAT-6 were manufactured by Peptide Protein Research Ltd, Wickham, England. The sequences of the peptides were as shown in figures 2.5-2.7.

2.2.3.3.1 Peptide stocks

Freeze-dried peptides were diluted in dimethyl sulphoxide (DMSO) at 10 mg/ml. Aliquots were stored at –80°C.

Peptide	Sequence
1	MQLVDRVRGAVTGMS
2	RVRGAVTGMSRRLVV
3	VTGMSRRLVVGAVGA
4	RRLVVGAVGAALVSG
5	GAVGAALVSGLVGAV
6	ALVSGLVGAVGGTAT
7	LVGAVGGTATAGAFS
8	GGTATAGAFSRPGLP
9	AGAFSRPGLPVEYLQ
10	RPGLPVEYLQVPSPS
11	VEYLQVPSPSMGRDI
12	VPSPSMGRDIKVQFQ
13	MGRDIKVQFQSGGAN
14	KVQFQSGGANSPALY
15	SGGANSPALYLLDGL
16	SPALYLLDGLRAQDD
17	LLDGLRAQDDFSGWD
18	RAQDDFSGWDINTPA
19	FSGWDINTPAFEWYD
20	INTPAFEWYDQSGLS
21	FEWYDQSGLSVVMPV
22	QSGLSVVMPVGGQSS
23	VVMPVGGQSSFYSDW
24	GGQSSFYSDWYQPAC
25	FYSDWYQPACGKAGC
26	YQPACGKAGCQTYKW
27	GKAGCQTYKWETFLT
28	QTYKWETFLTSELPG
29	ETFLTSELPGWLQAN
30	SELPGWLQANRHVKP
31	WLQANRHVKPTGSAV
32	RHVKPTGSAVVGLSM
33	TGSAVVGLSMAASSA
34	VGLSMAASSALTLAI
35	AASSALTLAIYHPQQ
36	LTLAIYHPQQFVYAG
37	YHPQQFVYAGAMSGL
38	FVYAGAMSGLLDPSQ
39	AMSGLLDPSQAMGPT
40.	LDPSQAMGPTLIGLA
41	AMGPTLIGLAMGDAG
42	LIGLAMGDAGGYKAS
43	MGDAGGYKASDMWGP
44	GYKASDMWGPKEDPA
45	DMWGPKEDPAWQRND
46	KEDPAWQRNDPLLNV
47	WQRNDPLLNVGKLIA
48	PLLNVGKLIANNTRV
49	GKLIANNTRVWVYCG
50	NNTRVWVYCGNGKPS
51	WVYCGNGKPSDLGGN
52	NGKPSDLGGNNLPAK
53	DLGGNNLPAKFLEGF
54	NLPAKFLEGFVRTSN
55	FLEGFVRTSNIKFQD
56	VRTSNIKFQDAYNAG
57	IKFQDAYNAGGGHNG
58	AYNAGGGHNGVFDFP
59	GGHNGVFDFPDSGTH
60	VFDFPDSGTHSWEYW
61	DSGTHSWEYWGAQLN
62	SWEYWGAQLNAMKPD
63	GAQLNAMKPDLQRAL
64	AMKPDLQRALGATPN
65	LQRALGATPNTGPAP
66	GATPNTGPAPQGA

Figure 2.5: Sequences of antigen 85A peptides

Peptide	Sequence
1	MTEQQWNFAGIEAAA
2	WNFAGIEAAASAIQG
3	IEAAASAIQGNVTSI
4	SAIQGNVTSIHSLLD
5	NVTSIHSLLDEGKQS
6	HSLLDEGKQSLTKLA
7	EGKQSLTKLAAAWGG
8	LTKLAAAWGGSGSEA
9	AAWGGSGSEAYQGVQ
10	SGSEAYQGVQQKWDA
11	YQGVQQKWDATATEL
12	QKWDATATELNNALQ
13	TATELNNALQNLART
14	NNALQNLARTISEAG
15	NLARTISEAGQAMAS
16	ISEAGQAMASTEGNV
17	QAMASTEGNVTGMFA

Figure 2.6: Sequences of ESAT-6 peptides.

Peptide	Sequence
1	MAEMKTDAATLAQEA
2	TDAATLAQEAGNFER
3	LAQEAGNFERISGDL
4	GNFERISGDLKTQID
5	ISGDLKTQIDQVEST
6	KTQIDQVESTAGSLQ
7	QVESTAGSLQGQWRG
8	AGSLQGQWRGAAGTA
9	GQWRGAAGTAAQAAV
10	AAGTAAQAAVVRFQE
11	AQAAVVRFQEAANKQ
12	VRFQEAANKQKQELD
13	AANKQKQELDEISTN
14	KQELDEISTNIRQAG
15	EISTNIRQAGVQYSR
16	IRQAGVQYSRADEEQ
17	VQYSRADEEQQQALS
18	ADEEQQQALSSQMGF

Figure 2.7: Sequences of CFP10 peptides.

2.2.3.3.2 *Peptide pools*

Peptides from the individual stocks were diluted in R0 medium to give a concentration of 50 μg/ml for each peptide in the pool. The peptide pools consisted of sequential peptides for CFP10 and ESAT-6, with the following peptides in each pool:

CFP10 - pool 1: peptides 1-6

pool 2: peptides 7-12

pool 3: peptides 13-18

ESAT-6 - pool 1: peptides 1-6

pool 2: peptides 7-12

pool 3: peptides 13-17

For Ag85A peptides, the pools were made up as shown in table 2.4.

Ag85A peptide pool						
A	B	C	D	E	F	G
1	3	5	7	9	11	13
15	17	19	21	23	25	27
29	31	33	35	37	39	41
43	45	47	49	51	53	55
57	59	51	63	65	2	3
6	8	10	12	14	16	18
20	22	24	26	28	30	32
34	36	38	40	42	44	46
48	52	54	56	58	60	62
64	66	50				

Table 2.4: Composition of Ag85A peptide pools.

(Numbers denote the peptide sequence as shown in figure 2.5).

2.2.4 Lymphocyte preparation

2.2.4.1 Isolation of peripheral blood mononuclear cells

Heparinised blood was diluted with approximately an equal volume of R0 medium before being layered over Ficoll-Paque PLUS (GE Healthcare, Buckinghamshire, England). Gradients were centrifuged at 400 *g* for 35 minutes at room temperature with the centrifuge brake switched off. The buffy coat containing the peripheral blood mononuclear cells (PBMC) was aspirated off using a fine-tipped pipette and diluted with 20 ml of R2 medium. PBMC were washed by spinning at 400 *g* for 10 minutes.

After removal of the medium, any remaining erythrocytes were lysed by resuspending in 5 ml filtered ACK lysis buffer (pH 7.2-7.4): 0.5 M ammonium chloride (Sigma), 1.0 mM potassium bicarbonate (Sigma), and 0.1 mM EDTA (Sigma). After 5 minutes, 20 ml of R2 medium was added and the cells washed by spinning at 400 *g* for 5 minutes.

The supernatant was removed, and the cell pellet dispersed in R2 medium for cell counting.

2.2.4.2 Isolation of lymphocytes from lymphoid tissue

Lymph nodes and spleen were supplied for immunological analysis at necropsy in dry sterile tubes. Tissues were put into a petri-dish with R0 medium. Any visible fat or connective tissue was cut away and removed. The lymphoid tissue was cut into small segments of approximately $<5mm^3$ size and placed into a medicon device (BD Biosciences, Oxford, England). R0 medium was added to the medicon before placing the

lid on and inserting it into a medimachine (BD Biosciences). After approximately 1 minute, the machine was turned off and the medicon removed. A Pasteur pipette was placed into the harvesting port and the single cell suspension was aspirated through the 50 μm filter contained within the medicon. The cell suspension was layered over Ficoll-Paque solution and gradients treated as described for PBMCs in section 2.2.4.1.

2.2.4.3 Depletion of lymphocyte subsets

Cells were depleted by adding magnetic beads coated with antibody (Dynal beads, Invitrogen, Paisley, England) against the cell subset to be depleted ($CD4^+$ or $CD8^+$). PBMCs were diluted to approximately $1x10^7$ cells/ml in PBS containing 2% FCS. Beads that had been pre-washed twice with PBS containing 2% FCS were added to cells at a ratio of 4 beads to 1 cell. The bead/cell suspension was incubated on a blood tube rotator for 30 minutes at 2-8°C.

Depleted cells were harvested by placing the tubes on a magnet (Invitrogen, Paisley, England) and leaving for 3 minutes to allow the beads to become fully attracted to the magnet. The cell suspension containing the depleted cell subset was then aspirated off and used in immunological analysis. Immunophenotyping analysis by flow cytometry confirmed successful depletion of cell populations.

2.2.4.4 Cryopreservation of lymphocytes

Cell suspensions were centrifuged at 400 *g* for 5 minutes. The supernatant was discarded and the cell pellet resuspended in 1 ml cryopreservation medium (90% fetal calf serum containing 10% DMSO) per vial to be frozen. Cells were frozen in 1 ml aliquots, each

containing between $8x10^6$-$1.2x10^7$ cells. Vials were placed in an isopropanol freezing device and kept at –80°C for at least 24 hours before being transferred to storage in liquid nitrogen vapour.

2.2.4.5 Resuscitation of cryopreserved lymphocytes

Cryopreserved cells were removed from storage in liquid nitrogen vapour and placed into a 37°C waterbath until thawed. The contents were then transferred to 10 ml R2 medium and the cells centrifuged at 400 *g* for 5 minutes. The supernatant was removed and the cell pellet resuspended in a known volume of R2 medium for counting.

2.2.5 Sera preparation

Blood was collected in serum separation tubes (BD Vacutainers) containing silica particles that activate clotting. After ensuring the clot has retracted by incubation at 2-8°C for up to 2 hours the tubes were centrifuged at 3,000 rpm for 10 minutes. The serum was collected and passed through a 0.45 μm filter followed by a 0.2 μm filter before being placed into labelled vials and stored at -20°C. Before use, samples of sera were heat-inactivated by placing in a 56°C waterbath for 45 minutes.

2.2.6 Quantification of a single cell suspension

Lymphocytes were counted using the trypan blue exclusion method. The cell suspension was diluted 1:10 with 0.4% trypan blue solution (Sigma) and mixed by pipetting up and down at least 10 times. The suspension was then loaded into a counting grid on a

disposable counting chamber (KovaSlide, Bio-Stat Ltd, Stockport, England). Cells were counted in the large squares in each of the four corners of the slide.

To calculate the number of cells, the mean number of cells for each corner square was determined. This was multiplied by 10^4, to account for the volume of cell suspension present in the counting area. The figure was multiplied by 10, to factor in the dilution factor of cell suspension with the trypan blue dye solution to give a cell count per ml. To determine the total cell count, the result was multiplied by the total volume of cell suspension.

2.2.7 Evaluating the frequency of interferon-γ secreting cells

The monkey/human IFN-γ kit (MabTech, Nacka, Sweden) was used in all ELISPOT assays. Sterile polyvinylidene difluoride (PVDF) plates (Millipore, Watford, England) were coated with 10 μg/ml of IFN-γ antibody (clone GZ-4) diluted in 0.1 M carbonate-bicarbonate buffer, pH 9.6 (Sigma-Aldrich) overnight at 4°C.

After incubation the coating antibody was removed and wells washed 6 times with 200 μl/well sterile PBS solution. Plates were then blocked by the addition of 100 μl per well of 0.45 μm-filtered R10 medium for 2 hours at 37°C with 5% CO_2.

Antigens and mitogens were diluted in R0 medium and 20 μl added to the appropriate wells. Cells were stimulated with antigens at the following final concentrations: PPD – 10 μg/ml; and 15mer peptides spanning Ag85A, CFP10 and ESAT-6 – 10 μg/ml (described in section 2.2.3). The assay also contained a negative and positive control. The negative control used medium alone and the positive control consisted of adding

phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) and ionomycin (CN Biosciences, Nottingham, England) at final concentrations of 100 ng/ml and 1 μg/ml, respectively.

100 μl cells suspended in R5 medium were then aliquoted into the wells at final concentrations of $2x10^5$, $1x10^5$ or $5x10^4$ cells/well. All tests were performed at least in duplicate, except medium alone where at least 8 replicate wells were used. Plates were incubated overnight between 17-20 hours at 37°C in a humidified atmosphere with 5% CO_2 undisturbed. Following this, plates were washed 6 times with 200 μl/well PBS + 0.05% Tween-20 (Sigma). 100 μl biotinylated anti-IFN-γ antibody (clone 7-B6-1) at 1 μg/ml diluted in PBS containing 0.5% filtered FCS was added to each well and incubated for 2 hours at 37°C.

Plates were washed 6 times with PBS containing 0.1% Tween-20. 100 μl streptavidin-ALP (1/1000 dilution made with PBS containing 0.5% filtered FCS) was added per well and incubated for 2 hours at room temperature.

Plates were washed 6 times with PBS containing 0.1% Tween-20 and then developed using 100 μl/well of 5-Bromo-4-Chloro-Indolyl Phosphate/Nitro Blue Tetrazolium (BCIP/NBT) substrate. Plates were left at room temperature for 20-40 minutes to allow spots to develop. To terminate the reaction, plates were washed three times with 200 μl/well of sterile water and dried at room temperature.

Well images were captured with an AID ELISPOT reader system and spots enumerated using the companion software (Autoimmun Diagnostika GmbH, Straβberg. Germany). Each well was manually checked to ensure consistency of spot counting.

Data were adjusted to give a value of spot-forming cells per 10^6 cells. This was done by subtracting mean counts seen in the media wells from the value obtained in the antigen wells from concentration-matched samples, and then multiplying this result to obtain a value equivalent to 10^6 cells. Where two different cell concentrations were used in the assay, each result was multiplied to 10^6 cells and then a mean value determined from both concentrations tested. If the number of IFN-γ-secreting cells was determined to be too numerous to count, the value per 10^6 cells was obtained from the lower concentration of input cells where spots could be quantified. Where the lowest concentration was also too high to count, a value of 250 spots/well was applied.

Where peptides for each antigen were tested in multiple pools, the spot-forming cells per 10^6 for each pool were added together to provide a total antigen response.

2.2.8 Measurement of cytokine concentrations after *in vitro* stimulation

2.2.8.1 Stimulation of blood and PBMC samples

Heparinised blood was diluted 1:10 with R0 medium, or where PBMC preparations were used, cells were diluted in R10 medium at a concentration of $1x10^6$ cells/ml. Antigens were tested at 5-10 μg/ml for PPD and approximately $2x10^4$ organisms per well for BCG (SSI). A negative control sample was cultured with R0 medium and a positive control stimulated in the presence of 5-10 μg/ml PHA (Sigma). Typically, 1 ml of blood/PBMC preparation was stimulated for each antigen/mitogen preparation using at least 6 wells of a 96-well U-shaped plate or in a 5 ml tissue culture tube.

After incubation the plates or tubes were centrifuged at 400 *g* for 5 minutes. The supernatants were passed through a 0.2 μm filter into sterile tubes and stored at –80°C until required for cytokine analysis.

2.2.8.2 Cytokine ELISA assays

2.2.8.2.1 IFN-γ ELISA

The human/monkey IFN-γ ELISA kit (MabTech) was used for measuring concentrations of IFN-γ in supernatant samples. Nunc maxi-sorb plates (VWR International Ltd, Leicestershire, England) were coated overnight at 4°C with 100 μl/well of 2 μg/ml of IFN-γ antibody (clone GZ-4) diluted in PBS.

Plates were washed 6 times with 200 μl/well of sterile PBS solution and blocked with 200 μl/well PBS containing 1% Bovine Serum Albumin (BSA) (Sigma-Aldrich) solution for 1 hour at room temperature.

Samples were thawed and vortexed before adding to the plate. 100 μl of each sample was added in duplicate wells. As a standard control, a purified human IFN-γ was tested in a 2-fold serial dilution across each plate starting at a concentration of 8000 pg/ml and diluted in incubation buffer: PBS containing 0.05% Tween-20 and 0.1% BSA. Samples were incubated overnight at 4°C.

Plates were washed 6 times with PBS containing 0.05% Tween-20 using an automated microplate washer (Molecular Devices, Berkshire, England). 100 μl/well of 1 μg/ml

biotinylated anti-IFN-γ antibody (clone 7-B6-1) diluted in incubation buffer was added. Plates were incubated for 1 hour at room temperature.

Plates were washed 6 times with 200 µl/well of PBS containing 0.05% Tween-20 and 100 µl/well of streptavidin (1/1000 dilution in incubation buffer) added to each well. This was incubated for 1 hour at room temperature.

Plates were washed a final 6 times with PBS containing 0.05% Tween-20 and 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate system (Sigma-Aldrich) added per well. This was left to develop at room temperature, typically for 5 minutes. The reaction was terminated by the addition of 30 µl 2M sulphuric acid solution (May & Baker Ltd, Dagenham. England).

Absorbances were read at 450 nm on a Multiskan EX microplate reader (Thermo LabSystems, Basingstoke, England) linked to analysis software (Genesis version 3.05, Thermo LabSystems). A standard curve was plotted from the dilutions of the IFN-γ control for each plate. From the standard curve, the absorbances obtained from the samples were evaluated to give the concentrations of IFN-γ.

2.2.8.2.2 IL-10 and IL-12 ELISA

Cytokine ELISA kits specific for monkey IL-10 and IL-12 were used (U-CyTech, Utrecht, The Netherlands). Nunc maxi-sorb plates (VWR International Ltd) were coated overnight at 4°C with 150 µl/well of coating antibody diluted in PBS. Plates were washed 6 times with 200 µl/well sterile PBS solution and blocked with 200 µl/well PBS + 1% Bovine Serum Albumin (BSA) (Sigma-Aldrich) solution for 1 hour at 37°C.

Samples to be tested were thawed and vortex mixed before adding to the plate. 100 µl of each sample was added in duplicate wells. As a standard control, purified monkey IL-10 and IL-12 was tested in a 2-fold serial dilution across each plate starting at a concentration of 30,600 pg/ml and 8,600 pg/ml, respectively. Dilutions were made with incubation buffer.

Plates were washed 6 times with 200 µl/well PBS containing 0.05% Tween-20 using an automated microplate washer (Molecular Devices). 100 µl/well biotinylated detector antibody diluted in incubation buffer was added. Plates were incubated for 1 hour at 37°C.

Plates were washed 6 times with 200 µl/well PBS containing 0.05% Tween-20 and 100 µl/well of Streptavidin-HRP polymer (SPP) conjugate added to each well. This was incubated for 1 hour at 37°C.

Plates were washed a final 6 times with 200 µl/well PBS containing 0.05% Tween-20 and 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate system added per well. This was left to develop at room temperature, typically for 15 minutes. The reaction was terminated by addition of 50 µl 2M sulphuric acid solution (May & Baker Ltd, Dagenham. England).

Absorbances were read at 450 nm, and analysed as shown for the IFN-γ ELISA assay (section 2.2.7.2.1).

2.2.8.2.3 Luminex assay

The luminex assay was used to measure multiple cytokines simultaneously in the same sample. The technology works by each cytokine antibody being conjugated to a bead set distinguished by its fluorescent intensity. The assay was set up in two parts: (i) microsphere binding; and (ii) running of samples and analysis.

2.2.8.2.4 Microsphere binding

50 µl of test sample was added to each well of a filter plate (Millipore). A standard dilution series of a cytokine control mixture was added in parallel to produce standard curves for all of the measured cytokines.

Each bead set coated with anti-cytokine antibody (Beadlyte system, Upstate, Dundee, UK) was made to a concentration of $1x10^5$ beads/ml, sonicated, and 25 µl added per well. Beadlyte® sets were used for IFN-γ, TNF-α, IL-2, IL-6 and IL-1β. The plate was covered with foil to protect from direct light and incubated on a plate shaker at room temperature for 90 minutes.

Each well was washed twice with 100 µl/well of assay buffer (PBS + 1% BSA + 0.05% Tween-20). A vacuum manifold was used to remove fluid via the base of the filter plate. 25 µl/well of 1 µg/ml reporter antibody (supplied as part of the Beadlyte system) was then added per well. Plates were incubated for 90 minutes at room temperature on a plate shaker in the dark.

25 µl of 40 µg/ml Streptavidin-Phycoerythrin was added to each well and incubated for 90 minutes on a plate shaker at room temperature. To each well, 25 µl stop solution (0.2% (vol/vol) formaldehyde in PBS) was added and left for 5 minutes on a plate shaker to fully mix. The plate was washed twice with 100 µl/well of assay buffer. Finally, the beads were resuspended in 100 µl/well of sheath fluid ready for acquisition on the luminex analyser.

2.2.8.2.5 *Acquistion of samples on the luminex and analysis*

Samples were analysed on a luminex-100 machine with StarStation software (Applied Cytometry Systems, Sheffield, England). For each sample, results were generated as shown in figure 2.8.

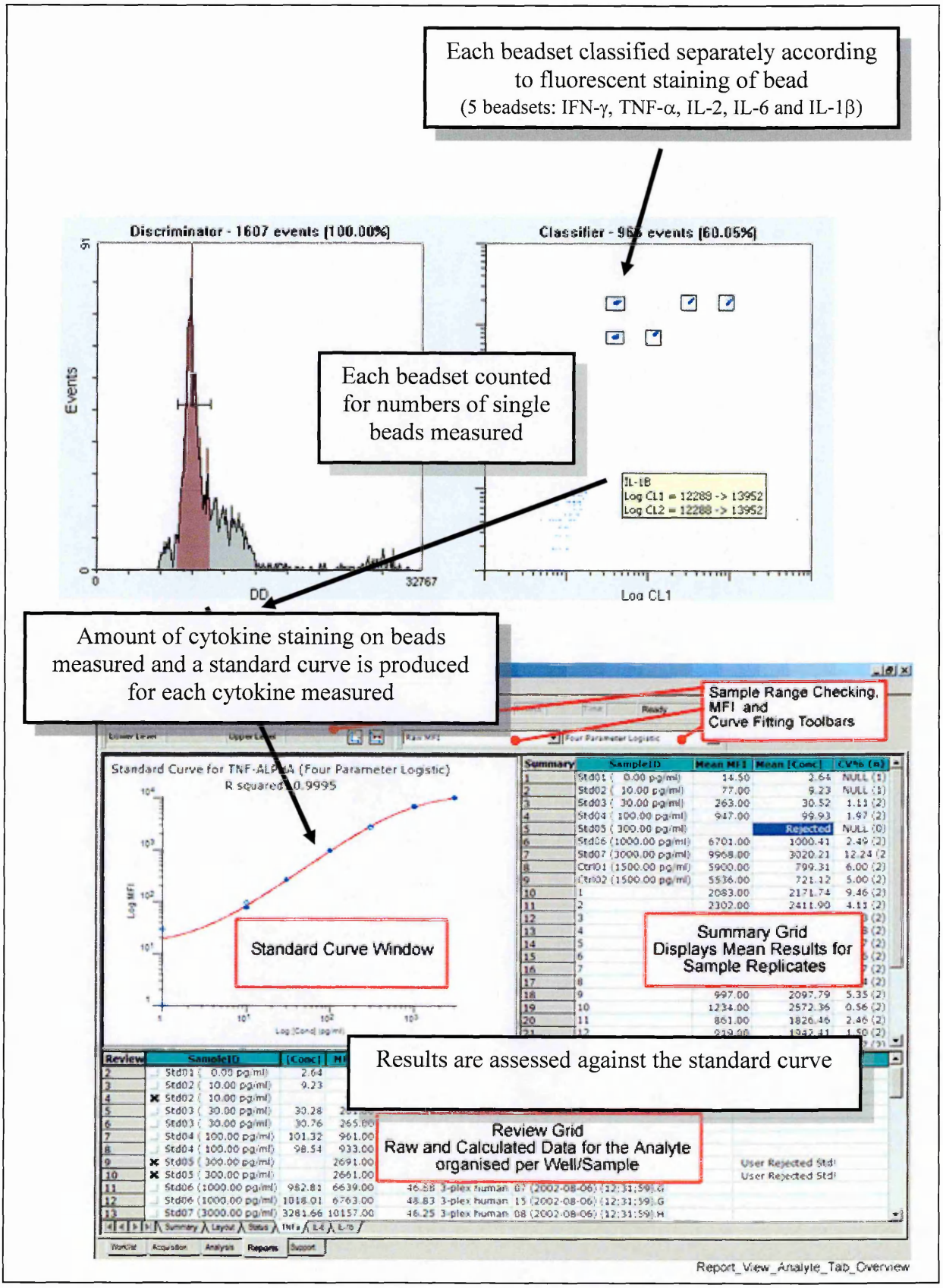


Figure 2.8: Overview of Starstation software used for luminex analysis

2.2.9 Vaccinia-specific antibody ELISA assay

To measure antibodies specific to the modified vaccinia Ankara (MVA) vector, a vaccinia-specific ELISA assay was used. MaxiSorp 96-well plates (Nunc Nalgene, Herefore. England) were coated with 100 µl/well of 2.5 µg/ml inactivated vaccinia virus Lister strain (Autogen Bioclear, England) diluted in carbonate coating buffer (0.015M Na_2CO_3 + 0.035M $NaHCO_3$ (Sigma) diluted in 1000 ml purified water). Plates were incubated at 2-8°C overnight.

Wells were washed three times with 350 µl/well trizma buffered saline (TBS) (0.14M NaCl + 0.002M KCl + 0.05M Tris + 0.009M Trizma HCl + 35 ml Brijl solution (Sigma) diluted in 20 L of purified water). Each well was blocked by the addition of 250 µl diluent buffer (5% skim milk powder (Sigma) diluted in PBS containing 0.01% Tween-20 (Sigma)) and left at room temperature for 1 hour.

Wells were washed three times with 350 µl/well TBS. Samples were diluted in duplicate four-fold down the plate in diluent buffer starting at 1:25. A control preparation of Vaccinia Immunoglobulin (VIG) starting at 8,500 IU/ml was also prepared on each plate. The bottom row of the plate was left as a negative control containing diluent buffer only. Plates were incubated at room temperature for 2 hours.

Wells were washed three times with 350 µl/well TBS. 100 µl/well of a 1/1250 dilution of anti-monkey IgG peroxidase (Insight Biotechnologies, England) was added per well. Plates were incubated at room temperature for 2 hours.

Wells were washed five times with 350 µl/well TBS. To each well 100 µl of 2,2`-azino-di[3-ethylbenzthiazolin sulphate] (ABTS) peroxidase substrate system (Insight Biotechnologies) was added. Plates were left for 25 minutes to allow the colorimetric reaction to develop. 100 µl/well of ABTS stop solution (Insight Biotechnologies) was added to terminate the reaction.

Absorbances were read using a VersaMax plate spectrophotometer linked to SOFTmax PRO software (Molecular Devices, England) using a wavelength of 405 nm.

To determine the units of VIG per ml the values where curves turn direction were ascertained from the SOFTmax PRO software, equating to the mid-point of the curve. The value from the test sample was divided by the value obtained for the VIG preparation and multiplied by 8,500 (the starting concentration of VIG).

2.2.10 Statistical analysis

Minitab (version 15) was used for all statistical analyses. A significance level of $P<0.05$ was used in all analyses.

When comparing groups of animals, the non-parametric Mann-Whitney U-test was used as the data were not consistently normally distributed. Where 10 or more points across a time-course were assessed using the Mann-Whitney statistical test, the Bonferonni correction was used to take into account multiple comparisons, and this lowered the threshold at which the p-value becomes signficant.

To analyse responses over a certain time-period the area under the curve analysis was measured using a function on SigmaPlot (version 10) that uses the Trapezium Rule. Resultant areas were then compared using the Mann-Whitney test.

In deciphering whether data from two sets were related, the Spearman's rank correlation coefficient (r_s) was used as data were not normally distributed.

3 DETERMINATION OF BASELINE IFN-γ RESPONSES

3.1 INTRODUCTION

This chapter provides details on IFN-γ responses measured in macaques before entering experimental schedules. By monitoring baseline levels, a limit above which responses could be designated as "positive" could be ascertained for use in future studies.

3.1.1 Mycobacteria-specific antigens

Having chosen to measure the frequency of cells secreting IFN-γ using an ELISPOT assay, and the concentration of IFN-γ secreted using an ELISA assay, the antigens used to stimulate the cells needed to be selected. *Mycobacterium* components able to stimulate T cells can broadly be divided into somatic antigens and secreted antigens. The majority of somatic antigens, described as potential ligands for the recognition of the infectious agent which include heat shock proteins, are derived from destroyed bacilli during the late phase of infection (Ulrichs *et al.*, 1998). In contrast, secreted antigens are produced by metabolically active organisms (Ulrichs *et al.*, 1998) and are largely implicated as targets for protective immune responses (Orme & Collins, 1984). The secreted antigens are processed and presented primarily in the context of MHC class II, and as the infection clears, the immune system then encounters a range of somatic antigens released from the degraded bacteria. This results in the stimulation of a broad T cell repertoire recognising all classes of antigen (Andersen, 1997). In experimental analysis, responses were measured using the following antigens: purified protein derivation (PPD), whole bacilli Calmette Guérin (BCG) organisms, antigen 85 (Ag85), 6 kDa early secretory antigenic target (ESAT-6) and culture filtrate protein 10 (CFP10).

3.1.1.1 Purified protein derivative

Purified protein derivative (PPD) is a crude, poorly defined mixture of mycobacterial antigens containing both secreted and somatic proteins (Andersen, 1997; van Pinxteren *et al.*, 2000b). For *in vitro* assays in this thesis, PPD was sourced from a commercial company, Statens Serum Institut (SSI), who also supply PPD for routine Mantoux testing in humans. The PPD used was derived from a culture of *M. tuberculosis*.

In addition to providing protein antigens for recall of adaptive T-cell responses, mycobacterial PPDs also elicit potent early-innate responses (Weir *et al.*, 2003). Due to being a protein lysate, it would be expected to be processed by the exogenous antigen presenting pathway resulting in presentation by MHC class II molecules and stimulation of $CD4^+$ T cells (Tesfa *et al.*, 2004). Due to the range of antigens contained in PPD, this antigen was used for the detection of responses against *M. tuberculosis* and BCG. Additionally, PPD is widely used as a mycobacteria-specific antigen by the scientific community for the detection of immune responses. This means that data can be compared between research groups, gives added confidence of its suitability in immunological assays, and allows for convenient simultaneous measurement of responses to a wide range of mycobacterial antigens.

3.1.1.2 BCG

Whole BCG organisms were derived from vaccine vials supplied by SSI. The BCG used in the *in vitro* assay was the same strain used for animal vaccinations and in human clinical trials conducted in Oxford.

BCG was only used in the whole blood stimulation assay that involved culture for 6 days, in parallel with human studies (Black *et al.*, 2001; Black *et al.*, 2002). BCG was not used as an antigen in the overnight ELISPOT assay due to less time for the organisms to undergo phagocytosis which subsequently leads to antigens being processed and presented effectively.

3.1.1.3 Antigen 85 complex

The proteins of the antigen 85 (Ag85) complex (Ag85A, Ag85B and Ag85C) are major secretion products of *M. tuberculosis* and *M. bovis* BCG (Wiker & Harboe, 1992). One of the functions of these proteins is to bind to fibronectin (Wiker & Harboe, 1992), which may aid intracellular mycobacterial survival and allow mycobacteria to bind to host cells aiding dissemination throughout host tissues (Armitige *et al.*, 2000). Others have reported that the Ag85 proteins act as enzymes called mycolyltransferases that are involved in the cell wall assembly in mycobacteria (Belisle *et al.*, 1997).

As one of the vaccines being tested in non-human primates in this thesis involved an MVA boost containing Ag85A, it was important to test responses against this mycobacterial protein. The MVA85A vaccine is currently in clinical trials in the UK and Africa (McShane *et al.*, 2005). The antigen was also chosen as it is present in all strains of BCG as well as being an immunodominant antigen in animal and human studies (McShane *et al.*, 2005).

3.1.1.4 Culture filtrate protein 10 and 6 kDa early secretory antigenic target

Culture filtrate protein 10 (CFP10) and 6 kDa early secretory antigenic target (ESAT-6) are both prominent T-cell targets during early TB infection (van Pinxteren *et al.*, 2000b). Their small size may make them more susceptible to proteolytic degradation and enhanced processing (Skjot *et al.*, 2000).

Genes encoding CFP10 and ESAT-6 are transcribed together in *M. tuberculosis* and both code for small exported proteins (Berthet *et al.*, 1998). Both genes are contained on the region of deletion 1 (RD1) section which is present in *M. tuberculosis* but absent in BCG strains (Behr *et al.*, 1999). CFP10 and ESAT-6 reside within one operon, indicating that they may be expressed and available for immune recognition in the same phase of infection (Brock *et al.*, 2004). It has been speculated that CFP10 and ESAT-6 may have a role in bacterial virulence (Skjot *et al.*, 2000), supported by the fact that they were deleted early in the process of attenuation of virulent *M. bovis* leading to the BCG vaccine (Harboe *et al.*, 1996).

Due to these antigens being absent in *M. bovis* BCG they can be used to differentiate between responses to active infection with *M. tuberculosis* compared to vaccination with BCG in IFN-γ assays. CFP10 alone has been reported to have a sensitivity of 70% in detecting *M. tuberculosis* infection using protein antigen (Dillon *et al.*, 2000). ESAT-6 has shown sensitivities of 65% (Ravn *et al.*, 1999) to 87% (Tesfa *et al.*, 2004); these differences may be due to separate patient populations being tested and also to variations in the nature of the antigen as the studies used protein preparations and peptides, respectively. When CFP10 and ESAT-6 antigens are combined, sensitivities in detecting

M. tuberculosis infection increased to 73% (van Pinxteren *et al.*, 2000b) using proteins or 93.1% (Scarpellini *et al.*, 2004) using selected immunogenic peptides.

It is important to note that genes encoding for CFP10 and ESAT-6 are present in environmental non-tuberculous mycobacteria (NTM) such as *M. kansasii, M. szulgai* and *M. marinum* (Skjot *et al.*, 2000). Therefore, prior exposure to these mycobacterial strains may contribute to CFP10- and ESAT-6-specific responses.

In this thesis, CFP10 and ESAT-6 antigens were used to determine responses post-challenge with *M. tuberculosis* so that responses detected would not be compromised by prior vaccination with BCG, i.e. would differentiate *M. tuberculosis* responses from those specific to BCG.

3.1.2 Chapter aims

The aims of the work described in this chapter were to ensure that assays and antigens were suitable for monitoring IFN-γ immune responses in non-human primates. This was achieved by applying the methods and antigens in a large cohort of naïve macaques involving 49 rhesus macaques and 18 cynomolgus macaques. This included all animals that entered the experimental studies described in this thesis, as well as other macaques for which samples were available.

Data from these analyses would also allow baseline levels of IFN-γ responses in naïve macaques to be determined. From these results, a cut-off limit could be elucidated to help define whether responses increased following subsequent vaccination and infection.

3.1.3 Chapter methods

Animals for TB work were screened using the PRIMAGAM kit for prior exposure to mycobacteria (section 2.2.1). The frequency of antigen-specific IFN-γ secreting cells was measured using an ELISPOT method (section 2.2.6) and the concentration of IFN-γ secreted after antigenic stimulation was measured using an ELISA method (section 2.2.7).

3.2 RESULTS

3.2.1 Primate IFN-γ (PRIMAGAM) test kit for *M. tuberculosis*

The commercial PRIMAGAM kit was used to determine whether animals had immune responses due to previous exposure to mycobacteria. Forty-seven rhesus macaques and 17 cynomolgus macaques were tested before being assigned to immunological studies. All animals screened were negative based on the manufacturer's guidance of a positive result of prior infection being indicated by an absorbance value of >0.05 that of the avian PPD- or negative antigen-stimulated samples.

3.2.2 Development of assays to measure IFN-γ

3.2.2.1 IFN-γ ELISPOT assay

3.2.2.1.1 Suitability of antigen preparations

In order to measure mycobacterium-specific cytokine responses using the ELISPOT assay, cells required stimulation with mycobacterial antigens. Suitable antigens should produce low background responses in naïve animals and induce specific responses in vaccinated or challenged animals.

3.2.2.1.1.1 Purified protein derivative

Purified protein derivative (PPD) is a complex mixture of antigens that are contained in both *M. tuberculosis* and *M. bovis* BCG. Responses to PPD were investigated in PBMCs isolated from naïve animals (49 rhesus macaques and 18 cynomolgus macaques) using the ELISPOT technique (section 2.2.6). All animals showed responses below 200 IFN-γ-producing cells per 10^6 PBMCs (<0.04%) in response to stimulation with PPD (figure 3.1). Animal-to-animal variation was observed, with the median response being 46 (range 0-131) and 33 (range 0-158) IFN-γ-producing cells/10^6 PBMC in rhesus macaques and cynomolgus macaques, respectively.

3.2.2.1.1.2 Recombinant proteins

Two recombinant proteins were investigated: antigen 85B (Ag85B) and ESAT-6. Both proteins were purified from *E. coli* expression vectors and were supplied from a commercial company (Lionex, Germany).

Responses to these recombinant proteins were assessed in PBMC from naïve animals: 33 rhesus macaques and 6 cynomolgus macaques. Responses were below 300 and 500 IFN-γ-producing cells per 10^6 PBMCs after stimulation with rAg85B and rESAT-6, respectively (figure 3.1). The median response to rAg85B was 87 (range 0-275) and 59 (range 0-192) IFN-γ-producing cells/10^6 PBMC in rhesus macaques and cynomolgus macaques, respectively. For rESAT-6, the median response in rhesus macaques was 97 (range 0-302) IFN-γ-producing cells/10^6 PBMC, and in cynomolgus macaques was 248 (range 0-491).

Responses to the two recombinant proteins (rAg85B and rESAT-6) in naïve macaques were compared with results observed after stimulation with PPD (figure 3.1). The data show that the responses to the recombinant proteins were significantly higher and more variable in naïve animals compared to those observed after stimulation with PPD.

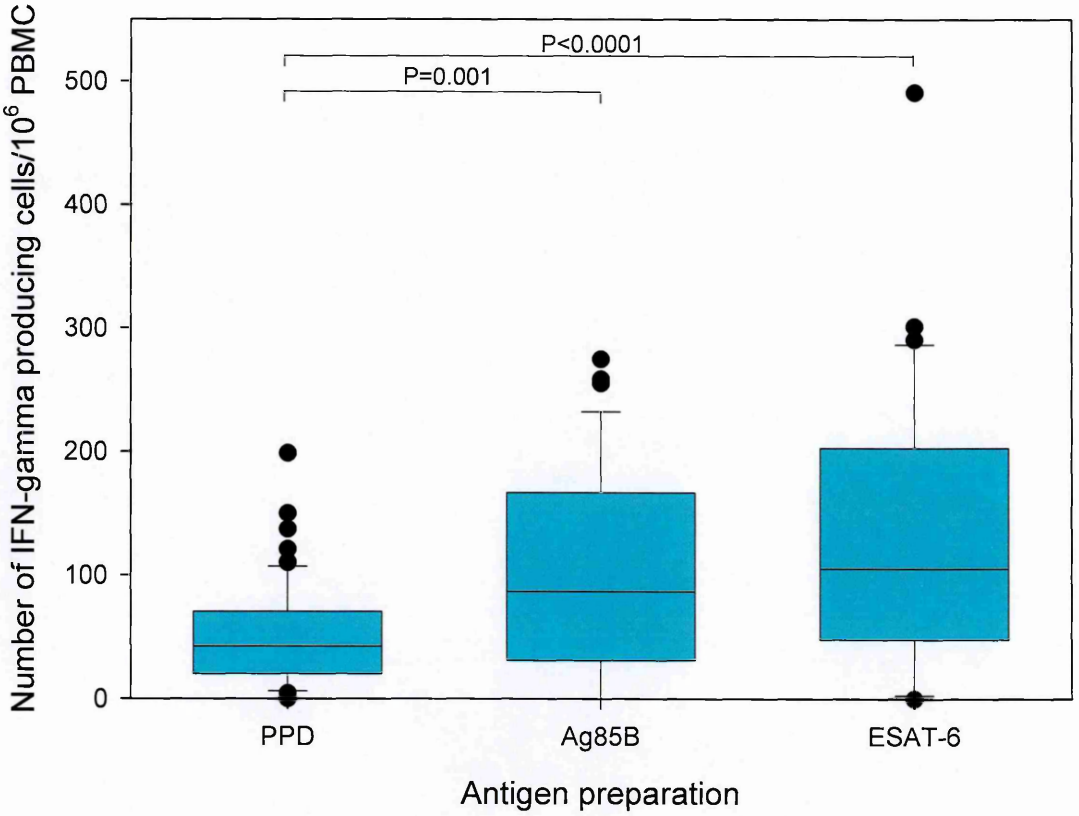


Figure 3.1: Box-plot comparison of different antigen protein preparations tested in naïve macaques using an IFN-γ ELISPOT assay.

(Each box encompasses 50% of the data. The median value is displayed as a line. The upper and lower bars of the box mark the limits of upper and lower quartile of the variable population. Whiskers extending from the top and bottom of each box mark the 10% and 90% limits. Outliers are displayed as individual points. Mann-Whitney statistical test, significance level $P<0.05$).

The unexpectedly high background responses seen to recombinant proteins in some of the animals could have been due to either the purity of the antigen preparation or prior exposure of the animals to mycobacterial antigens, possibly as a result of frequent tuberculin skin testing to ensure animals remained free from mycobacterial disease. Responses to ESAT-6, which is present in *M. tuberculosis* but absent in BCG, is indicative of TB infection. However, the data from prior testing with the diagnostic PRIMAGAM kit using PPD (which also contains ESAT-6) was negative and animals showed no clinical signs of infection (e.g. weight loss, coughing). Also, the PPD ELISPOT responses were low in these animals, thus providing further evidence of a lack of mycobacterial infection and evidence that frequent skin testing is not the reason for high baselines in some animals.

To determine if these high background responses were due to the immune status of cells isolated from the animals, responses to rESAT-6 and rAg85B were measured in cryopreserved PBMC collected from naïve rhesus macaques obtained from 3 different breeding colonies. Two of the colonies were based in the UK (Dstl and Oxford) and one in China. The UK breeding colonies carried out tuberculin testing at a much reduced interval (once prior to delivery) than the Chinese colony where animals were tested routinely every 6 months.

Additionally, in order to determine if the *E. coli* expression system used to prepare the recombinant Ag85B and ESAT-6 proteins was responsible for the high background values, responses to Simian Immunodeficiency Virus (SIV) proteins prepared in a baculovirus system (gp130) and an *E. coli* system (p27) were included in the assay. The SIV proteins were sourced from the AIDS repository held at the National Institute for Biological Standards and Controls (NIBSC).

Results from this experiment showed that the pattern of responses was similar in animals from each of the different colonies (figure 3.2). The *E. coli* derived proteins produced consistently high responses whether they were TB proteins or SIV proteins. However, the baculovirus-derived protein induced a much lower response equivalent to that seen to medium alone.

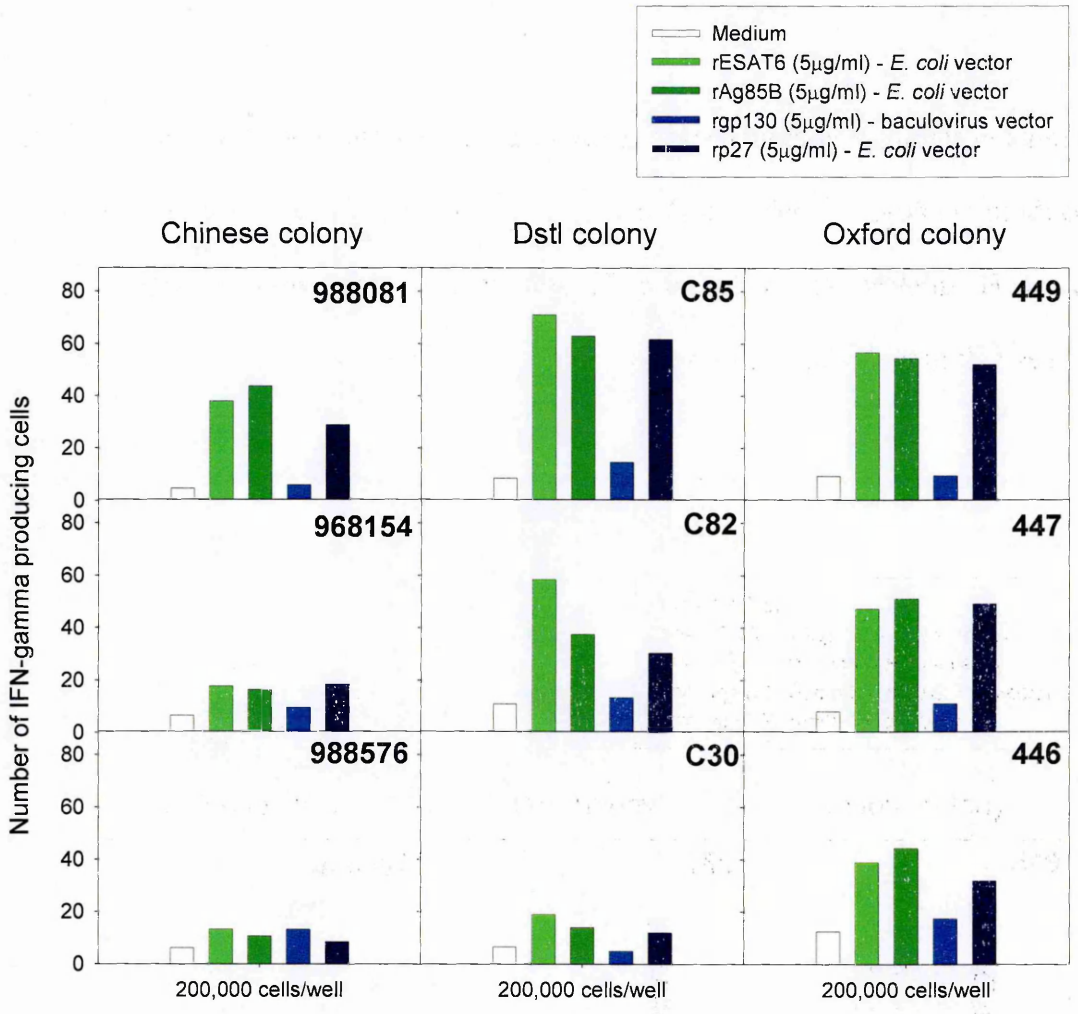


Figure 3.2: Responses to TB and SIV proteins in PBMC from naïve rhesus macaques obtained from different breeding colonies.

(All results were analysed in the same assay using cells that had been cryopreserved. The number in each box refers to the unique identifier for each animal. TB proteins, green bars; SIV proteins, blue bars).

3.2.2.1.1.3 Peptide pools

Overlapping 15mer peptides corresponding to gene sequences from *M. tuberculosis* H37Rv for Ag85A (66 peptides), ESAT-6 (17 peptides) and CFP10 (18 peptides) were manufactured (Peptide Protein Research Ltd, England). Peptides were pooled: 7 pools for Ag85A, 3 pools for ESAT-6, and 3 pools for CFP10.

To compare the peptide pools with proteins, a common antigen (ESAT-6) between both preparations was used. Direct comparisons were carried out in 37 naïve animals; 31 rhesus macaques and 6 cynomolgus macaques. Results show that the background responses were generally lower using peptide pools (figure 3.3). However, there were some animals where responses were higher using peptide pools than with the protein. Overall, the mean number of IFN-γ-producing cells/10^6 PBMC was 115 after protein stimulation, and 44 after stimulation with peptide pools (a 61.7% reduction). When all of the results were compared, this reduction was statistically significant (paired t-test, P=0.006).

For subsequent experiments, peptide pools were used in addition to PPD.

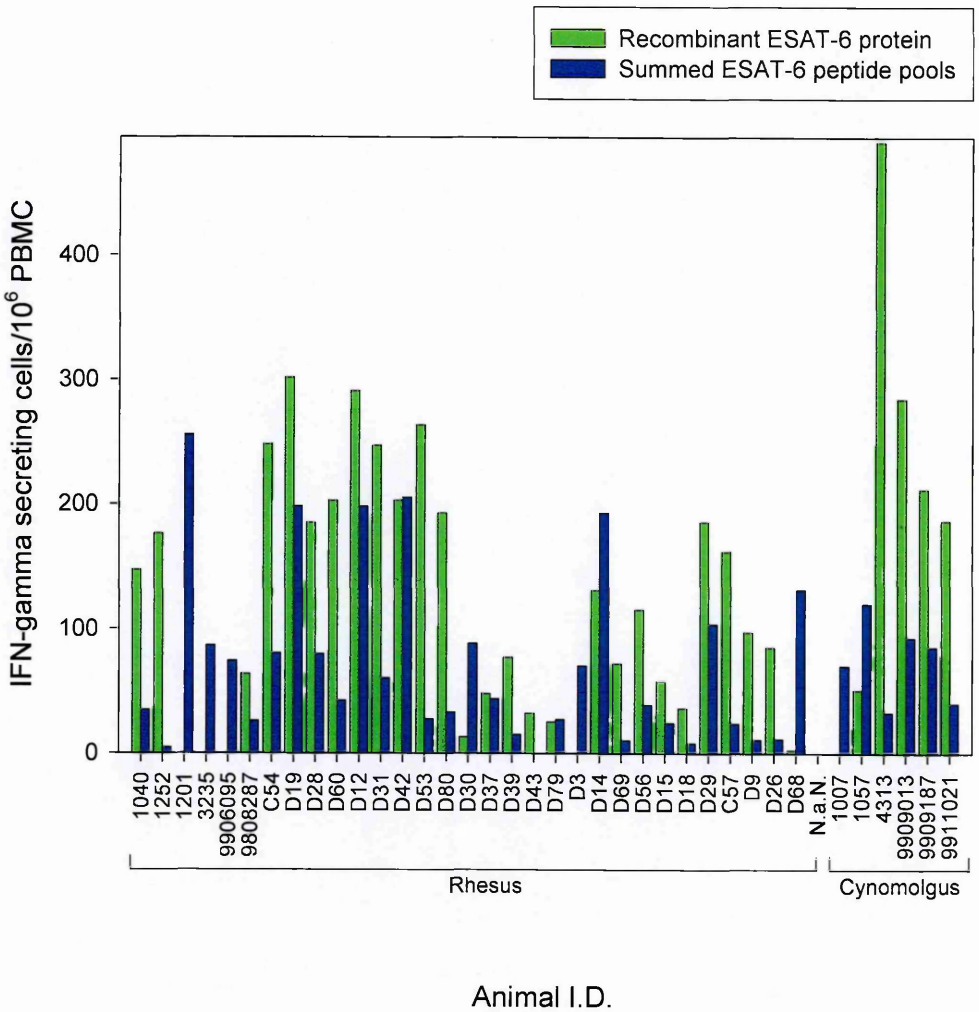


Figure 3.3: Comparison of background IFN-γ ELISPOT responses to ESAT-6 using recombinant protein and peptide pools as antigens in naïve macaques.

(Recombinant protein, green bars; peptide pools, blue bars).

3.2.2.1.2 Macaque species range for IFN-γ ELISPOT antigens in naïve animals

Having tested PPD and the peptides pools (Ag85A, CFP10 and ESAT-6), the ranges for IFN-γ-secreting cells/10^6 PBMC from rhesus macaques and cynomolgus macaques were ascertained for all four antigens (table 3.1). As shown in figure 3.4, most of the antigens showed an interquartile range below 100 spots per 10^6 PBMC (<0.0001%). However, the responses to Ag85A were higher and showed greater variability.

Due to the range of responses in the macaque populations, determining a baseline response for use in experimental studies could not rely on using an average population value for each species. Therefore, responses in individual animals were investigated in further detail.

	Macaque species	IFN-γ-secreting cells/10^6 PBMC	
		Median	Range
PPD	Rhesus (n=49)	46	0-131
	Cynomolgus (n=18)	33	0-158
Ag85A peptides	Rhesus (n=48)	96	0-1436
	Cynomolgus (n=17)	33	0-386
CFP10 peptides	Rhesus (n=47)	30	0-331
	Cynomolgus (n=17)	14	0-90
ESAT-6 peptides	Rhesus (n=47)	68	0-284
	Cynomolgus (n=17)	33	0-93

Table 3.1: Summary of IFN-γ responses to mycobacterial PPD and peptide pools in naïve macaques.

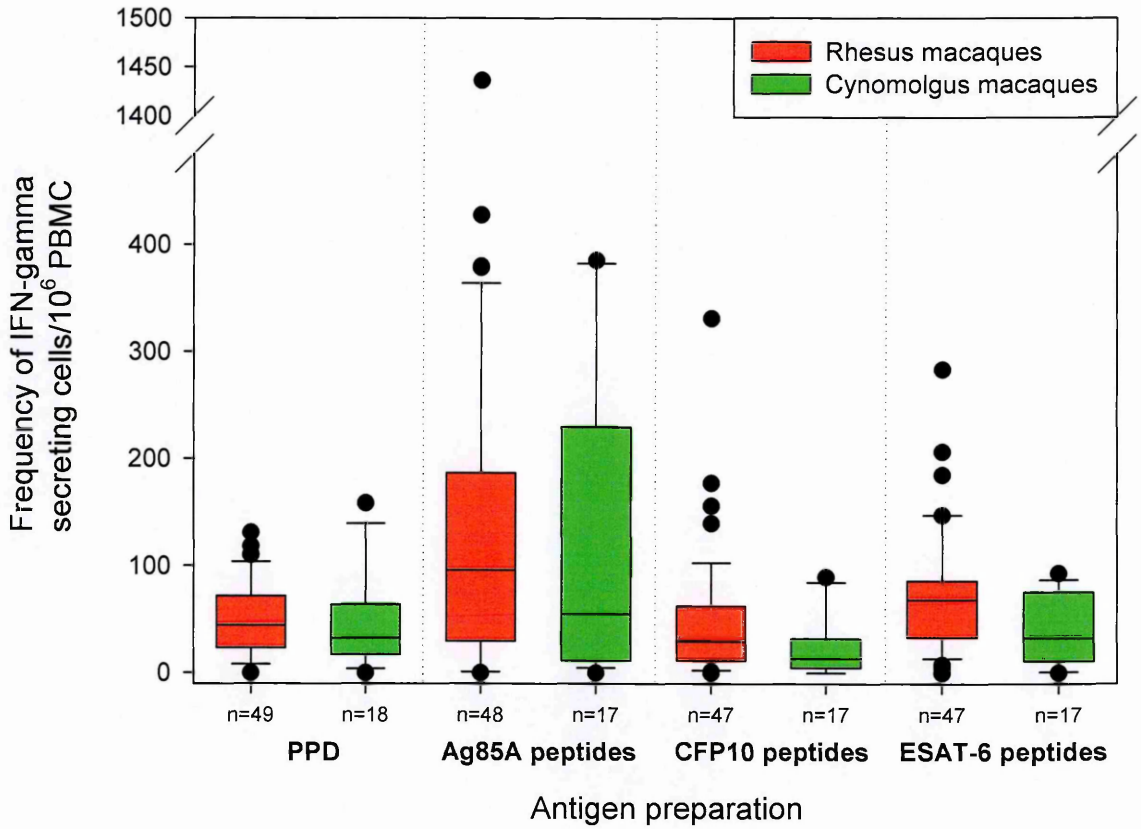


Figure 3.4: Macaque population responses to PPD and peptide pools in naïve animals. (Each box encompasses 50% of the data. The median value is displayed as a line. The upper and lower bars of the box mark the limits of upper and lower quartile of the variable population. Whiskers extending from the top and bottom of each box mark the 10% and 90% limits. Outliers are displayed as individual points).

3.2.2.1.3 Individual range for IFN-γ ELISPOT antigens in naïve macaques

Where baseline responses had been assessed on multiple occasions, the variability in response for each individual animal was determined (figures 3.5-3.8). Error bars represent 95% confidence intervals (CI). It should be emphasised that with some of the animals baselines were only measured on two occasions whereas, with others, responses were tested at up to 14 different time-points. However, there was no apparent difference in variation between samples tested twice and those on more occasions.

The background levels after stimulation with Ag85A peptide pools were higher than those for the other three antigens: PPD and peptides pools to CFP10 and ESAT-6. Levels and variation were similar between the rhesus macaques and the cynomolgus macaques.

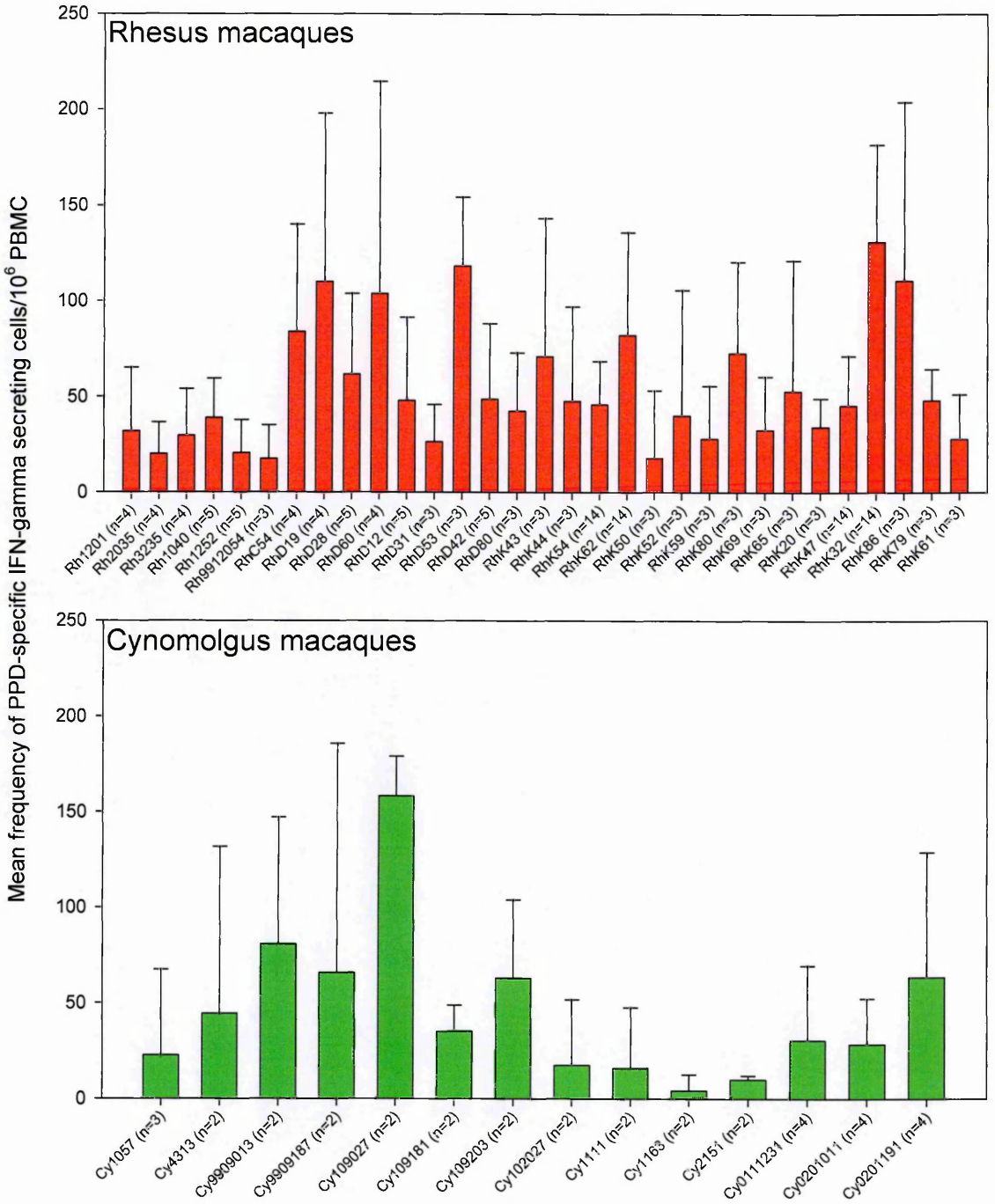


Figure 3.5: Variations in frequencies of PPD-specific IFN-γ -secreting cells in naïve macaques.

(Rhesus macaques, red bars; cynomolgus macaques, green bars. Errors bars show 95% CI. n = the number of occasions on which individual macaques were tested).

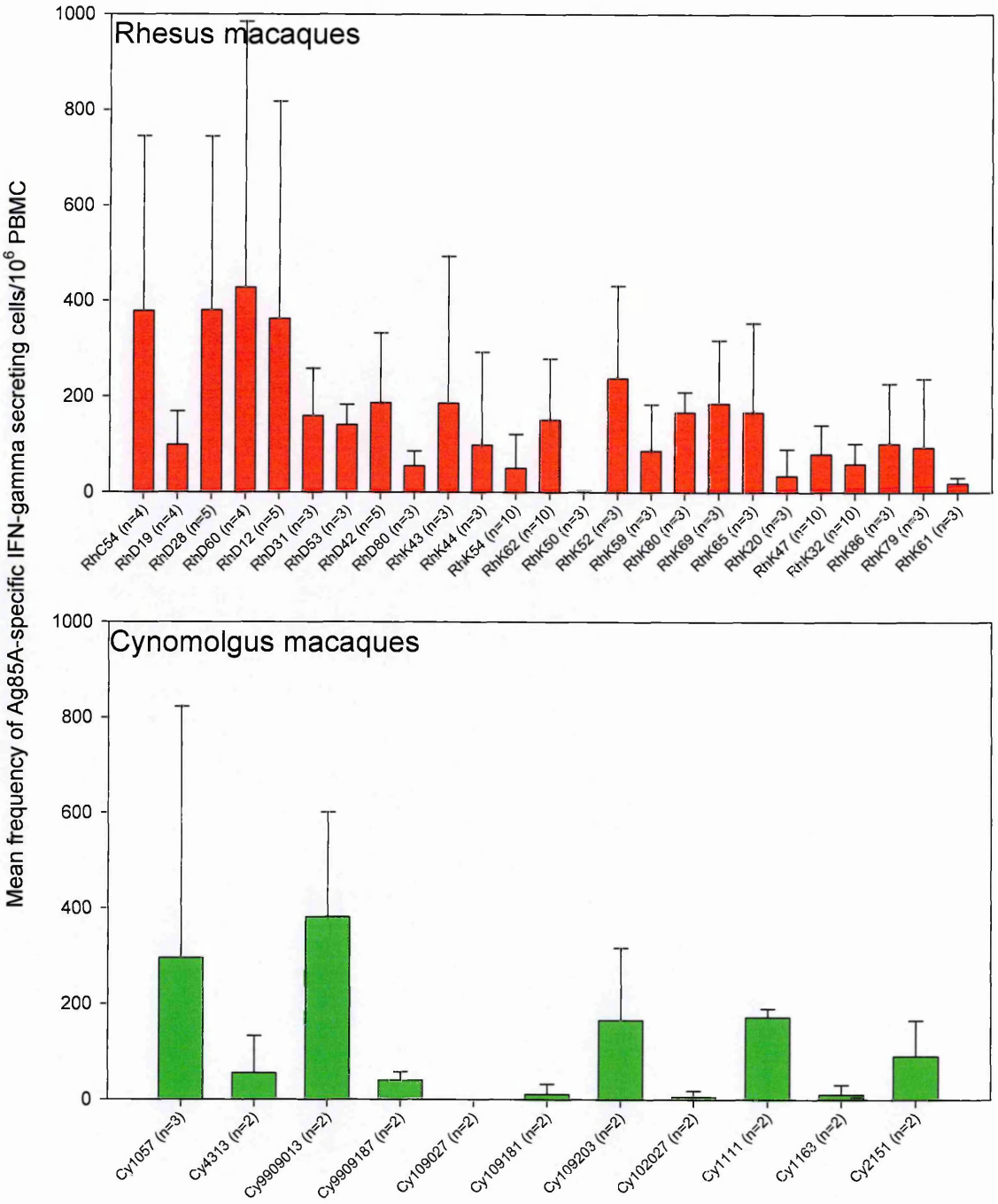


Figure 3.6: Variations in frequencies of Ag85A-specific IFN-γ -secreting cells in naïve macaques.

(Rhesus macaques, red bars; cynomolgus macaques, green bars. Errors bars show 95% CI. n = the number of occasions on which individual macaques were tested).

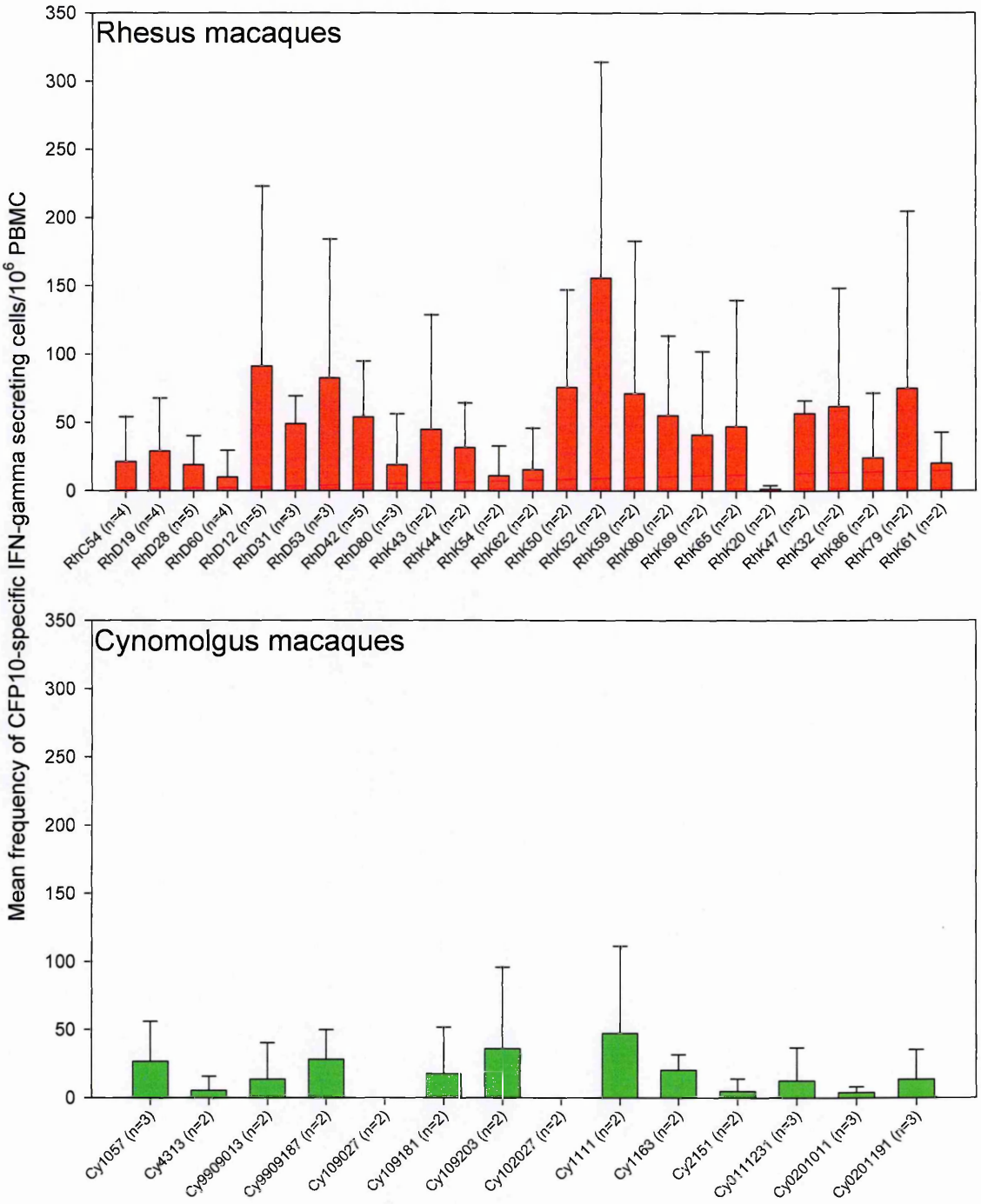


Figure 3.7: Variations in frequencies of CFP10-specific IFN-γ -secreting cells in naïve macaques.

(Rhesus macaques, red bars; cynomolgus macaques, green bars. Errors bars show 95% CI. n = the number of occasions on which individual macaques were tested).

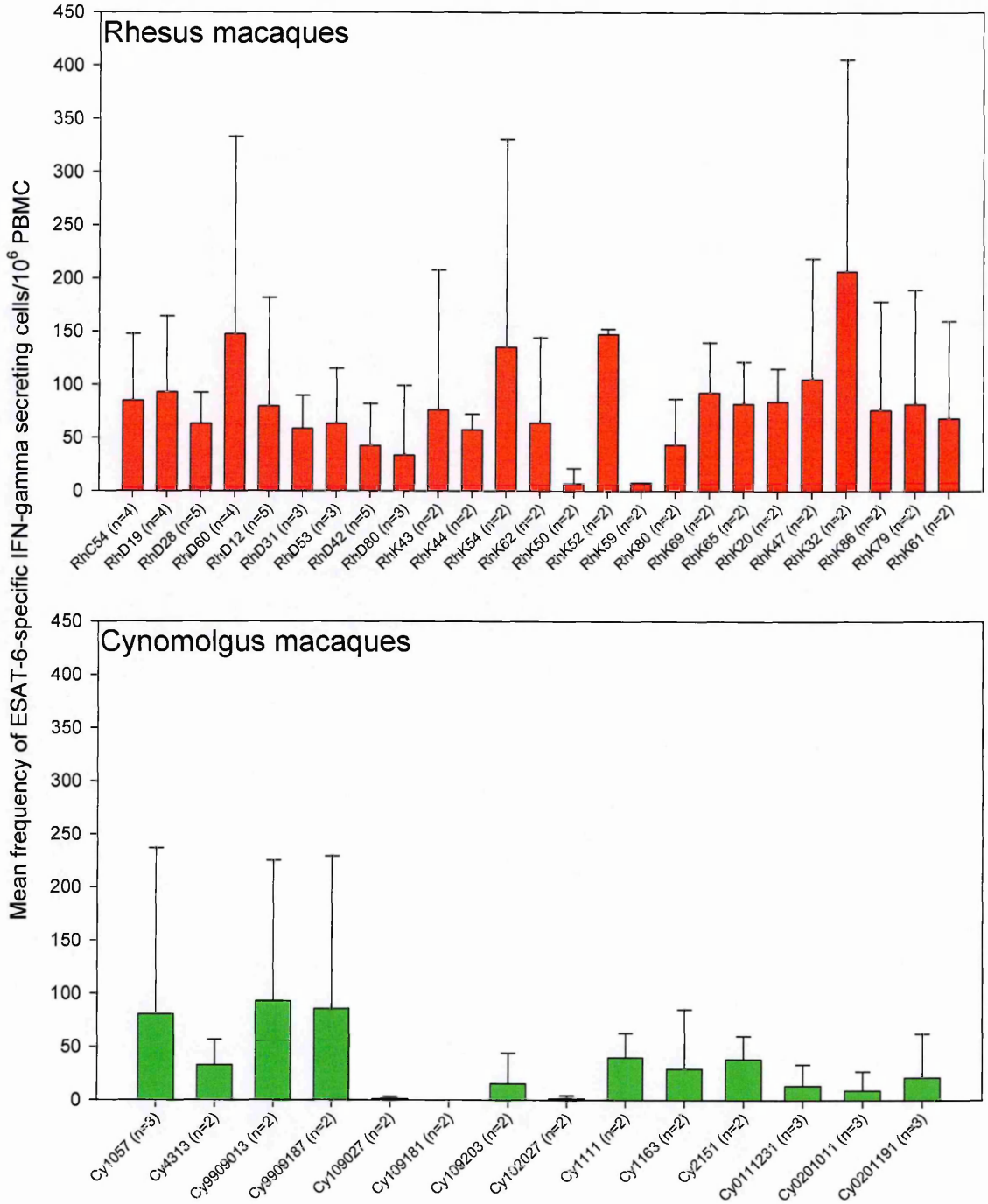


Figure 3.8: Variations in frequencies of ESAT-6-specific IFN-γ -secreting cells in naïve macaques.

(Rhesus macaques, red bars; cynomolgus macaques, green bars. Errors bars show 95% CI. n = the number of occasions on which individual macaques were tested).

3.2.2.1.4 Determination of a positive IFN-γ ELISPOT response

Due to the range of variation between individual animals, a cut-off limit for each animal was required in order to define a positive IFN-γ ELISPOT response. Using the 95% confidence intervals for each animal, the upper limit was expressed as a multiplication factor of the mean (figure 3.9).

When each antigen was plotted, it became apparent that on no occasions did the upper 95% confidence interval extend to over 3 times the mean response. Therefore, **a positive ELISPOT response would be defined in future studies as being over 3 times the average baseline level.**

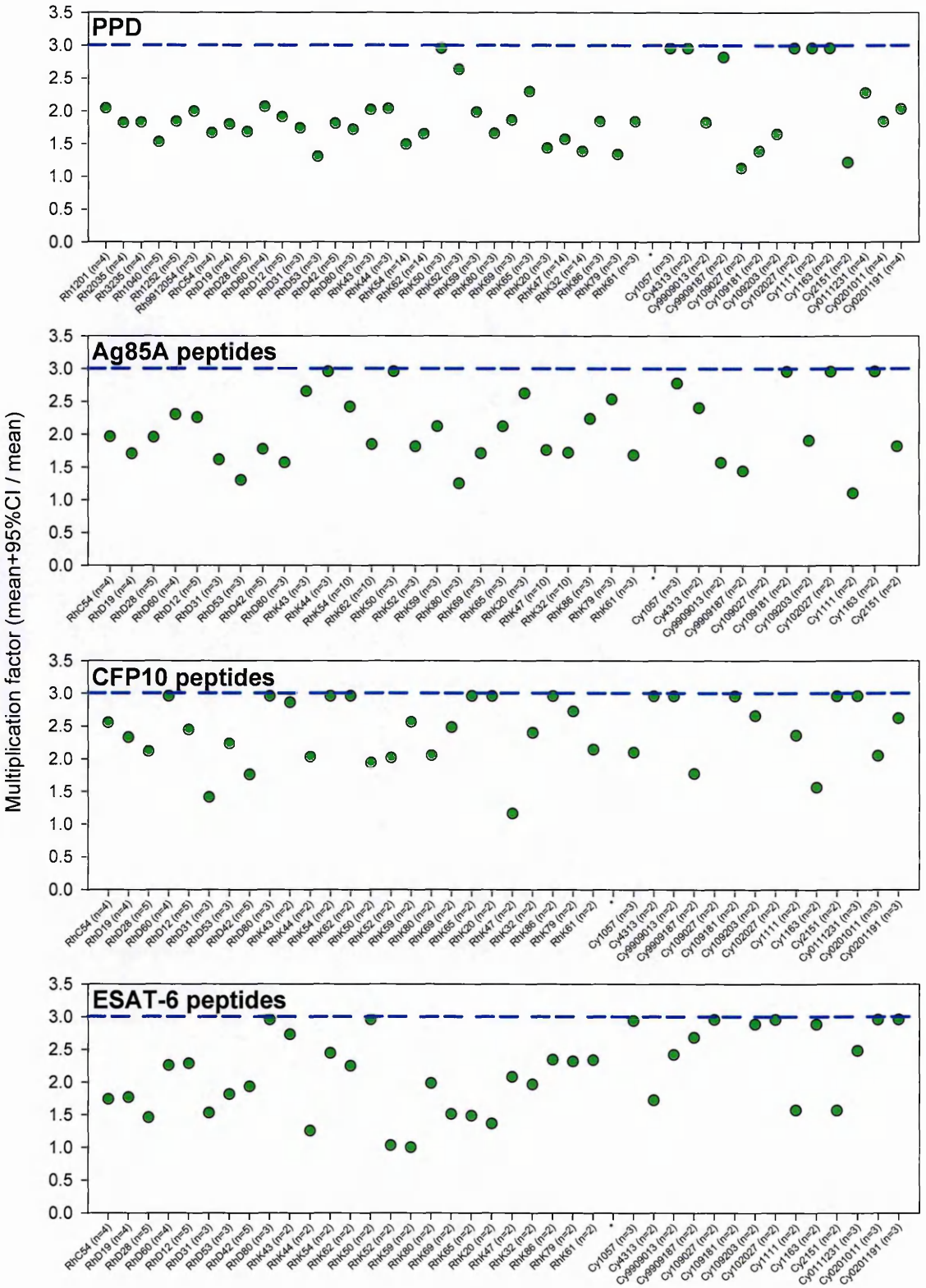


Figure 3.9: Multiplication factor of the mean baseline level needed to obtain the upper 95% confidence interval during baseline screening of macaques.

(Rh; rhesus macaques: Cy; cynomolgus macaques).

3.2.2.2 Development of the whole blood ELISA assay

The ELISA assay used to measure IFN-γ in diluted whole blood supernatants was carried out using a commercially-available monkey IFN-γ-specific kit.

During the stimulation assay, two antigens were tested: PPD and BCG. In naïve samples, the background levels of IFN-γ secretion was measured after 6 days of stimulation. As shown in figure 3.10, only one animal exceeded secretion of 100 pg/ml with the majority of animals having undetectable levels of IFN-γ. When the groups of animals were compared, there was a higher concentration of IFN-γ secreted in rhesus blood after stimulation with BCG than after stimulation with PPD. However, this difference was not observed with cynomolgus macaques.

As the background levels of antigen-specific IFN-γ secretion were low, this assay was used in subsequent experiments without modification. **The cut-off limit in defining a positive IFN-γ ELISA response was determined to be 100 pg/ml.**

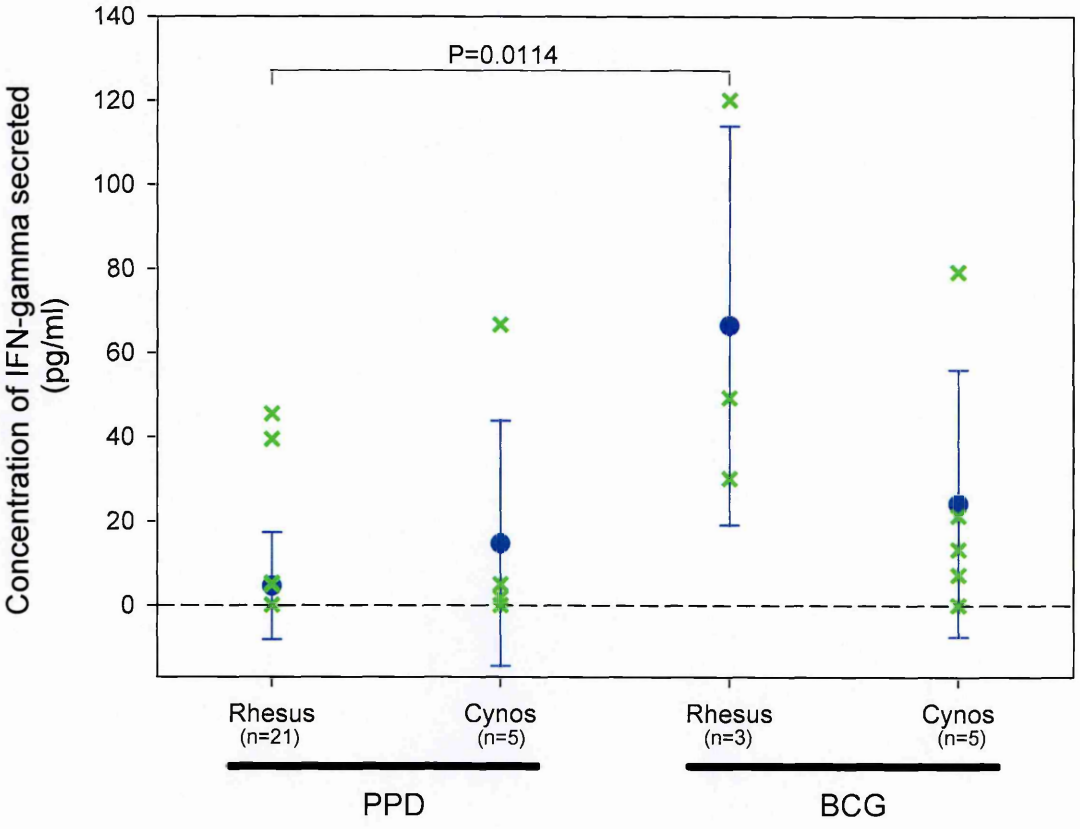


Figure 3.10: Levels of antigen-specific IFN-γ secretion measured by ELISA in naïve macaques.

(Blue error bars denote the mean +/- standard deviation. Green crosses denote values for individual animals).

3.3 DISCUSSION

3.3.1 Screening of animals

Prior to the measurement of any immune responses in non-human primates, all animals were screened for any potential *M. tuberculosis* infection or prior exposure using a commercially-available IFN-γ based whole blood assay kit (PRIMAGAM). This kit had been used to screen a colony of rhesus and cynomolgus macaques during an outbreak of *M. bovis*, and was reported to have a 68% sensitivity (Garcia *et al.*, 2004). However, at the Biomedical Primate Research Centre (BPRC) in The Netherlands the PRIMAGAM kit was used on 23 experimentally-infected animals (15 cynomolgus macaques and 8 rhesus macaques) and results from the test were positive for all animals (Vervenne *et al.*, 2004). It should be noted that in the BPRC study the infection was artificially-induced as it was achieved through intratracheal inoculation of $1x10^5$-$3x10^5$ cfu of *M. tuberculosis*, which is a large dose and is administered in a way that does not mimic natural infection; therefore, all animals would be expected to generate large positive immune responses.

The advantages of using an *in vitro* assay as compared to the traditional tuberculin skin test are: the ability to repeat tests without interfering with the animal's immune system; less time is required to gain results; a larger range of antigens can be included; and the procedure is more comfortable and less invasive to the animals. However, it should be noted that a negative *in vitro* immunological test for TB in primates does not necessarily indicate that animals are free of *M. tuberculosis*. As no "gold-standard" exists for diagnosis of TB in macaques, the PRIMAGAM test was used to ensure that to the best of knowledge no prior exposure had occurred. In addition, animals were also monitored for clinical signs of disease (e.g. weight loss, x-ray, bacteriology), with no indicators of TB being found in any of the naïve animals.

3.3.2 Development of the IFN-γ ELISPOT assay

3.3.2.1 Assessment of baseline responses

With all of the antigens tested, individual animal-to-animal variation was observed. Marked inter-subject variability in homeostatic levels of circulating antigen-specific T cells (Comin-Anduix *et al.*, 2006) means that ideally multiple baseline samples should be routinely collected as part of an immunisation schedule (Cox *et al.*, 2006). Although this is not always feasible in routine practice (Comin-Anduix *et al.*, 2006), for non-human primate studies the opportunities do exist to sample routinely before experimental studies begin. The differences between animals could be caused by different antigenic expression levels due to genetically-based characteristics of antigen processing and immune responsiveness in the non-human primates, including different cytokine secretion profiles or polymorphisms of human leukocyte antigen (HLA) or cytokine receptors (Arend *et al.*, 2000a).

3.3.2.2 Use of PPD as an antigen

IFN-γ ELISPOT assay responses to PPD in naïve macaques were generally low (<200 antigen-specific IFN-γ-producing cells/10^6 PBMC). In humans, baseline levels of PPD-specific responses have been reported in 3 unvaccinated, tuberculin skin-test negative donors of 13.33-30 IFN-γ-producing cells/10^6 PBMC (Helms *et al.*, 2000). Though the same antigen concentration was used, 10 μg/ml, the source of PPD was different in the two studies. The cause of the background response to PPD may be through either cross-stimulation with non-mycobacterial infection or priming of T cells with an opportunistic

mycobacterial infection, such as *M. avium* (Helms *et al.*, 2000). Given that animals were all deemed negative by the PRIMAGAM kit this latter explanation is unlikely.

Both the ELISPOT and PRIMAGAM kit use PPD as antigen, but responses were detected in ELISPOT when PRIMAGAM results were negative. It could be that differences in methodologies might account for differences in response. Both assays measure responses after overnight stimuation; however, the PRIMAGAM uses whole blood and measures the concentration secreted, whereas the ELISPOT uses PBMC to measure the number of cells secreting IFN-γ. Importantly, PPD for the PRIMAGAM assay was manufactured from *M. bovis* whereas the PPD used in the ELISPOT assay was derived from *M. tuberculosis*. Variability might be expected as different batches of PPD have been reported to have varying protein concentrations and antigenic profiles (Tameni *et al.*, 1998). Alternatively, the concentration of PPD is 10 μg/ml in the ELISPOT assay, whereas for the PRIMAGAM the concentration is approximately 85 μg/ml (Prionics AG, personal communication). Therefore, it is impractical to compare results from PRIMAGAM and ELISPOT assay, despite both using PPD as antigen. It was likely that due to the high sensitivity of the ELISPOT assay the responses that the assay detected were naturally circulating levels of reactive cells.

As background responses to PPD in the ELISPOT assay were low, and due to this antigen containing a variety of mycobacterial antigens, PPD was included as an antigen for subsequent ELISPOT assays.

3.3.2.3 Recombinant proteins

Recombinant ESAT-6 and Ag85B proteins were also tested in the IFN-γ ELISPOT assay. The advantage of using whole protein antigens was that each antigen could be tested in fewer wells. Also, as proteins will need to be processed they may further mimic the natural environment. However, soluble protein antigens primarily stimulate $CD4^+$ T cells (Gomez *et al.*, 2004) meaning that $CD8^+$ responses could potentially be missed (Tesfa *et al.*, 2004).

High background responses were observed using these protein antigens. Further analysis on cryopreserved cells of naïve animals from different breeding colonies appeared to show that repeated skin testing was not the cause. Repeated skin testing in humans and the induction of false positive results in IFN-γ assays is controversial, with some reports saying there is no effect (Richeldi *et al.*, 2006) and other studies showing the opposite (Vilaplana *et al.*, 2008). However, as all of the proteins (TB and SIV) produced in *E. coli* showed high levels of IFN-γ-producing cells, whereas the baculovirus-derived protein did not, the expression vector seemed the probable cause of this response in naïve macaques.

The cause of the increased frequency of IFN-γ-producing cells might be due to endotoxin contamination of the protein preparations. This lipopolysaccharide (LPS) component of the outer cell membrane of *E. coli* (and other Gram negative organisms) has a large number of deleterious biological activities (Fraser, 1982), and is active in nanogram amounts (Ozaki *et al.*, 1989). With their multiple potent effects on biological systems, endotoxins represent a possible source of variability in experimental biology and pathology (Fumarola, 1981). Therefore, to prevent experimental artefacts in biological

assays it is crucial to remove or at least significantly reduce LPS levels in protein preparations (Salek-Ardakani *et al.*, 2002). The use of Gram positive organisms, such as *Bacillus subtilis* and various *Streptomyces* species as host organisms, may help to overcome the problem with endotoxin (Fraser, 1982).

Recombinant ESAT-6 has been used in immunological assays after depletion of residual endotoxin through an LPS affinity column (Arend *et al.*, 2000b) or by producing ESAT-6 in other vectors, such as *Lactococcus lactis* (Ewer *et al.*, 2003). Alternatively, polymixin B, a potent antibiotic that binds to and neutralises LPS, has been utilised so that *E. coli* produced recombinant proteins can be used in cytokine assays (Cardoso *et al.*, 2007). The proteins in the above example were contaminated with 0.132-0.210 ng/ml LPS (Cardoso *et al.*, 2007). The level of endotoxin in the rESAT-6 preparation used in the ELISPOT assay in the current study was 456 ng/ml. The endotoxin level of the rAg85B protein preparation was not available. Other investigators have also had concerns with *E. coli* preparations and have instead investigated the baculovirus expression system (Williams *et al.*, 1998).

Soluble protein antigens have been used before for different pathogens. In primate studies against *Leishmania major*, soluble *Leishmania* antigen was used and gave background responses of <15 spots/10^6 cells (Freidag *et al.*, 2003). In humans, recombinant protein of tick-borne encephalitis virus has been used as a neoantigen, with background responses of under 300 spots per million cells (Gomez *et al.*, 2004), though the PBMCs were stimulated with protein for 3 days before being transferred to ELISPOT plates for assaying.

For future experiments, peptides were assessed in place of recombinant proteins. This was partly to overcome the problems encountered with the increased background

responses, but mainly due to peptides being used in parallel human studies conducted in Oxford. By using the same antigen preparations, responses would then be comparable between human responses and those detected in macaques reported in this thesis.

3.3.2.4 Peptide antigens

Peptides have the advantage over whole protein in that instead of biasing towards $CD4^+$ T cells, they are also capable of inducing $CD8^+$ T cell responses (Freidag *et al.*, 2003). As they do not require internal processing an overnight incubation is usually sufficient to allow direct binding to MHC molecules on the surface of cells (Mashishi & Gray, 2002), with no advantage to using longer incubation times up to 48 h (Kumar *et al.*, 2001; Russell *et al.*, 2003).

The length of peptides can vary. Peptides of 8-12 amino acids (aa) produce the highest levels of $CD8^+$ responses, 20 aa peptides bias more towards the $CD4^+$ subset, and 15 aa peptides are effective with both $CD4^+$ and $CD8^+$ T cells (Maecker *et al.*, 2001). Although 15 aa peptides should be too long to properly fit into the MHC class I binding groove the investigators suggested that: (1) it is possible that 15mers bound to some degree without properly fitting into the binding groove; (2) the peptides were shortened by extracellular proteases; (3) the peptides were actually internalised and processed prior to binding to class I MHC; and (4) synthesised intermediates consisting of shorter peptides that contaminate the final peptide preparation might be responsible (Maecker *et al.*, 2001). Therefore, length of peptides is determined by which responses are to be detected and other practicalities; for example, the use of 15 aa peptides is a balance between testing a feasible number of peptides and detecting both $CD4^+$ and $CD8^+$ responses (Maecker *et al.*, 2001; Mashishi & Gray, 2002; Mwau *et al.*, 2002). Additionally, using 15 aa

peptides has shown over 50% increases in frequencies of IFN-γ-producing cells compared to 20mers, suggesting that 15mers increase the ability to detect low-level responses (Russell *et al.*, 2003), likely to be due to more efficient presentation via the MHC molecule due to the smaller size.

We used pools of 15mer peptides to measure responses to Ag85A, CFP10 and ESAT-6. As both the protein and peptides for ESAT-6 were available it was possible to make a direct comparison with the two antigen preparations. A 62% reduction in background response was seen using the peptides, most likely due to the absence of endotoxin in the peptide preparations. Peptide pools to CFP10 and ESAT-6 have been used by other investigators and shown to give similar results as whole proteins in proliferation and PBMC-stimulated ELISA assays (Arend *et al.*, 2000b), although in this example peptides were 20 aa in length. However, the same observation has also been reported for 15 aa peptides using ICS analysis (Maecker *et al.*, 2001). Therefore, the use of peptides can mirror responses seen when using protein preparations.

The background responses to the peptide pools for CFP10, ESAT-6 and Ag85A were low with <331, <284 and <450 (except one animal, where the baseline response was 1450) IFN-γ-secreting cells/10^6 PBMC, respectively. When peptides of 9-10 aa in length have been used in cancer patients specific for melanoma, cytomegalovirus and Epstein-Barr virus, the detection limit has been reported to be 70 IFN-γ spots per million PBMC (Comin-Anduix *et al.*, 2006). However, in that study primed antigen-presenting cells were added to PBMC so the assay was not conducted in the same way as described for the macaque studies. When 20 aa malarial peptides were used, background counts have been reported in the range of 10-50 IFN-γ spots/10^6 PBMC in rhesus macaques (Kumar *et al.*, 2001). The background responses with the TB peptides in macaques are higher

than those reported in the studies described above. The difference with the human study could be due to the species, with macaques having intrinsically higher background levels of responding cells. Alternatively, differences in peptides may be the cause, possibly due to cross-reactive antigenic regions on the peptides causing an increased baseline response. There have been examples where peptides have given high background responses, but this was due to incomplete dissolving of peptide causing a reaction with the avidin component of the detection system (Karlsson *et al.*, 2004). To overcome this phenomenon, we dissolved peptide stocks in DMSO before further diluting them out in tissue culture medium. The final DMSO concentration was always kept below 1% to avoid cellular toxicity (Russell *et al.*, 2003).

3.3.2.5 Positive controls

For the IFN-γ ELISPOT assay we used PMA and ionomycin as a positive control. The use of this type of control confirms that the assay was performed correctly (Currier *et al.*, 2002) by controlling the staining procedure (Mashishi & Gray, 2002). PHA has also been used as a mitogen in the ELISPOT assay. As with PMA and ionomycin, the spots after stimulation with PHA vary widely in size, intensity and shape; and due to the high spot number per well, they can also reach confluence (Currier *et al.*, 2002; Janetzki *et al.*, 2004). These mitogens typically induce the secretion of cytokine from more than one subset of cells, using mechanisms other than antigen recognition (Janetzki *et al.*, 2004). After 4 h stimulation with PMA and ionomycin, the frequency of IFN-γ-producing cells rises from <1% to 12%, reaching 19% by 12 h (Rostaing *et al.*, 1999). Stimulation with mitogen indicated that the cells were viable, and if responses were to be quantified the input cell number would need to be decreased compared with the rest of the assay plate (Mwau *et al.*, 2002).

Additional controls that are relevant to antigen presentation have been used in human studies, including pools of peptides from cytomegalovirus, Epstein Barr and influenza virus (CEF pool) recognised by $CD8^+$ T cells and presented by 11 class I HLA-A and HLA-B alleles whose cumulative frequencies represent the majority of Caucasian individuals (Currier *et al.*, 2002). The CEF pool produced spots that were comparable with experimental results, thus providing a standard for spot definition, especially when using an automated reader system (Janetzki *et al.*, 2004). However, these peptides are human HLA-specific and a non-human primate alternative was not available.

3.3.2.6 Enhancement of the ELISPOT assay

Cells can be cultured and stimulated with antigen prior to analysing them in the ELISPOT assay in order to determine lower frequencies of cytokine-secreting cells. Seven day culturing has been used to detect low-level responses in multiple sclerosis patients (McCutcheon *et al.*, 1997). Others have cultured for ten days to detect longer-lived memory T cells in malaria-vaccinated volunteers (Keating *et al.*, 2005) and for pre-screening of mycobacterial responses before entering vaccine studies (McShane *et al.*, 2004). For our IFN-γ ELISPOT assay we wanted to directly detect *ex vivo* responses to determine as close as possible the *in vivo* response without altering the kinetics of the cell populations by prior incubation and stimulation. Therefore, the overnight assays would represent IFN-γ production by differentiated T cells (Sirriyah *et al.*, 2004).

After development, plates were counted with an automated ELISPOT reader by the same operator. This eliminates discrepancy between individual operators, which has been shown to vary considerably (Ryan *et al.*, 2005), especially in wells containing higher

numbers of spots (Janetzki *et al.*, 2004). Although counting plates using the computer settings has proved to be the least variable method of plate counting (Janetzki *et al.*, 2004), the presence of artefacts and non-inclusion of some of the spots made it necessary to revisit each well to confirm results, as also recommended by others (Mwau *et al.*, 2002).

3.3.2.7 Defining the cut-off value for the IFN-γ ELISPOT

Results gained from the analysis undertaken in naïve rhesus and cynomolgus macaques showed that baseline responses varied. Therefore, defining a constant cut-off value specific for the particular macaque species may obscure positive responses from being recorded in animals with low backgrounds or result in false positive results. Instead, an individual cut-off for each animal would need to be defined.

Where serial baseline measurements were recorded, mean values and 95% CI were calculated. From these results, it was seen that the upper 95% CI never exceeded the mean baseline value by more than threefold. It was decided that the cut-off value for all subsequent experiments would be 3 times the value of the average pre-bleed. This would be particularly relevant where responses were low, giving increased confidence that responses were "real" and not merely naturally fluctuating baseline levels.

Others who use similar methodologies in human studies have used a cut-off defined of being at least twice that of negative control wells and above 5 (Beveridge *et al.*, 2008) or 30 (Brookes *et al.*, 2008) spot-forming units/10^6 cells. Alternatively, a cut-off of 17 spot-forming units/10^6 PBMC has been applied to ELISPOT studies in human trials (Hawkridge *et al.*, 2008).

3.3.3 Development of the whole blood IFN-γ ELISA assay

To eliminate discrepancies between the ELISA and ELISPOT assays, the same antibody pairs were used to measure IFN-γ. The coating antibody clone GZ-4 and detector antibody clone 7-B6-1 have been recommended as being optimal for non-human primate IFN-γ samples (Lazarevic *et al.*, 2005b; Makitalo *et al.*, 2002).

The ELISA assays used supernatants prepared from whole blood or PBMC. Whole blood is deemed to be the best medium in which to study cytokine production (De Groote *et al.*, 1992). IFN-γ ELISA results are also comparable if blood processing is delayed, although sensitivity does decrease after periods as short as 2 h (Doherty *et al.*, 2005). The whole blood assays were carried out soon after collection of samples, thus ensuring the minimal effect of any time delay.

As the diluted whole blood ELISA assay is being used in human clinical trials, the assays used in macaques were as similar as possible so that results would be comparable. The only optimisation carried out was to increase the incubation time of the sample in the ELISA assay from two hours at room temperature to overnight at 2-8°C. The presence of various binding proteins and other factors in plasma/serum may interfere with antibody binding (Prabhakar *et al.*, 2002) so the time was increased to allow for all of the available IFN-γ to bind to the detector antibody. This proved not to affect results from the recombinant standards giving confidence that the assay was not compromised by increasing the time from 2 h at room temperature to overnight at 4 °C (appendix 2, section 8.2).

Background levels of IFN-γ production after PPD stimulation in naïve macaques was always below 100 pg/ml. The same was true when using BCG as an antigen, with the exception of one animal where the level was ≈120 pg/ml. Therefore, a cut-off limit for defining a positive IFN-γ ELISA response was set at 100 pg/ml. Studies conducted in humans have defined a positive responses as being greater than 62 pg/ml (Beveridge *et al.*, 2008; Black *et al.*, 2001; Weir *et al.*, 2008).

3.4 CONCLUSION

Using a large cohort of naïve macaques, variation in responses was seen in individual animals of both species. Cut-off values were used to define a positive response (ELISPOT: >3 times the mean pre-bleed values; ELISA: >100 pg/ml), which allows a reproducible and standardised criteria to permit IFN-γ responses to be measured post-infection with *M. tuberculosis* (chapter 4) and after vaccination with BCG and other TB vaccines (chapter 5).

4 IFN-γ RESPONSES DURING *M. TUBERCULOSIS* INFECTION

4.1 INTRODUCTION

The dynamics of the immune response were studied in macaques following aerosol infection prior to investigating immune responses after vaccination. For newly developed TB vaccines, the probability of success could be significantly enhanced through a better understanding of the main steps in the pathogenic pathways to bacillary pulmonary TB (Wiegeshaus *et al.*, 1989). Therefore, the main objective of the work described in this chapter was to monitor and analyse IFN-γ responses post-infection with *M. tuberculosis* to identify the role of IFN-γ in the disease process.

4.1.1 Human studies of *M. tuberculosis* infection

Data on immune responses during the early stages of *M. tuberculosis* infection in humans are rarely available. This is because most reports of immune responses are undertaken in patients with advance stages of disease, and also the time since exposure is usually not known.

There are two documented studies where the time since exposure was known. One involved an accident in a microbiological laboratory where technicians were potentially exposed to *M. tuberculosis* (Leyten *et al.*, 2006). However, in the two individuals who converted to being tuberculin skin test (TST) positive, one was BCG vaccinated. This therefore does not reflect a naïve individual. The first samples from both individuals were taken 4 months after the accidental exposure and both had been treated with an antimycobacterial drug. In a second study, a large point-source TB outbreak at a school

in the UK was described, where the prolonged infectious period of the cases was known (Ewer *et al.*, 2006). This presented a unique opportunity to track *M. tuberculosis*-specific T-cell responses longitudinally in a cohort of contacts recently exposed to the same strain of *M. tuberculosis* (Ewer *et al.*, 2006). In this study, 11 staff were monitored who did not receive antimycobacterial treatment and became TST-positive during the 18 month follow-up period; these latently-infected subjects had continual levels of circulating RD1-specific IFN-γ-secreting cells.

Due to the lack of information on early immune responses after *M. tuberculosis* infection in humans, the non-human primate model provides an unique opportunity to measure temporal changes in immune dynamics during the early stages of disease in animals that very closely resemble humans in anatomy and physiology.

4.1.2 Aerosol challenge of non-human primates

The ideal challenge route for animal models should mimic the situation in human infection. Therefore, the best available option in studying TB infection requires aerosolisation of *M. tuberculosis*. Macaques challenged with a few airborne organisms accurately reflects infection in humans under natural conditions (Barclay *et al.*, 1970). Using the aerosol route with very small numbers of bacilli produces a limited, but not overwhelming disease (Barclay *et al.*, 1973).

Many researchers using non-human primates have used bronchoscopes to instill *M. tuberculosis* into the lung airways in rhesus macaques (Good, 1968; Gormus *et al.*, 2004; Langermans *et al.*, 2001; Schmidt, 1972) and cynomolgus macaques (Capuano *et al.*, 2003; Flynn *et al.*, 2003; Fuller *et al.*, 2003; Langermans *et al.*, 2001; Langermans *et al.*, 2005; Walsh *et al.*, 1996). Instillation of *M. tuberculosis* via bronchoscope is usually

performed on one lung, thereby not giving an even distribution of bacilli as occurs via delivery through respiratory inhalation. Additionally, using this challenge method, the bacilli may not reach into the alveolar macrophages as occurs in natural infection.

As alveolar macrophages are primarily found in the alveoli, to reach these levels deep in the lung the bacterial suspension needs to be effectively inhaled. In the early 1970s researchers used aerosol infection of rhesus macaques (Anacker *et al.*, 1972; Barclay *et al.*, 1970; Barclay *et al.*, 1973; Janicki *et al.*, 1973), but in-depth immunological analyses were not performed in these studies. If the bacteria do not reach the alveolar macrophages, then it is likely that other macrophages or dendritic cells (DC) will present antigen. DCs are found widely within the bronchopulmonary tree in both humans and mice (Sertl *et al.*, 1986). However, even though aerosolised bacilli will reach the alveolar macrophages, DCs still reside in the lung and may still be among the first cells to encounter the mycobacteria upon infection (Henderson *et al.*, 1997). Therefore, stimulating both DCs and alveolar macrophages will mimic human infection and allow for the induction of relevant cytokine immune responses (Fenton & Vermeulen, 1996; Serbina & Flynn, 1999).

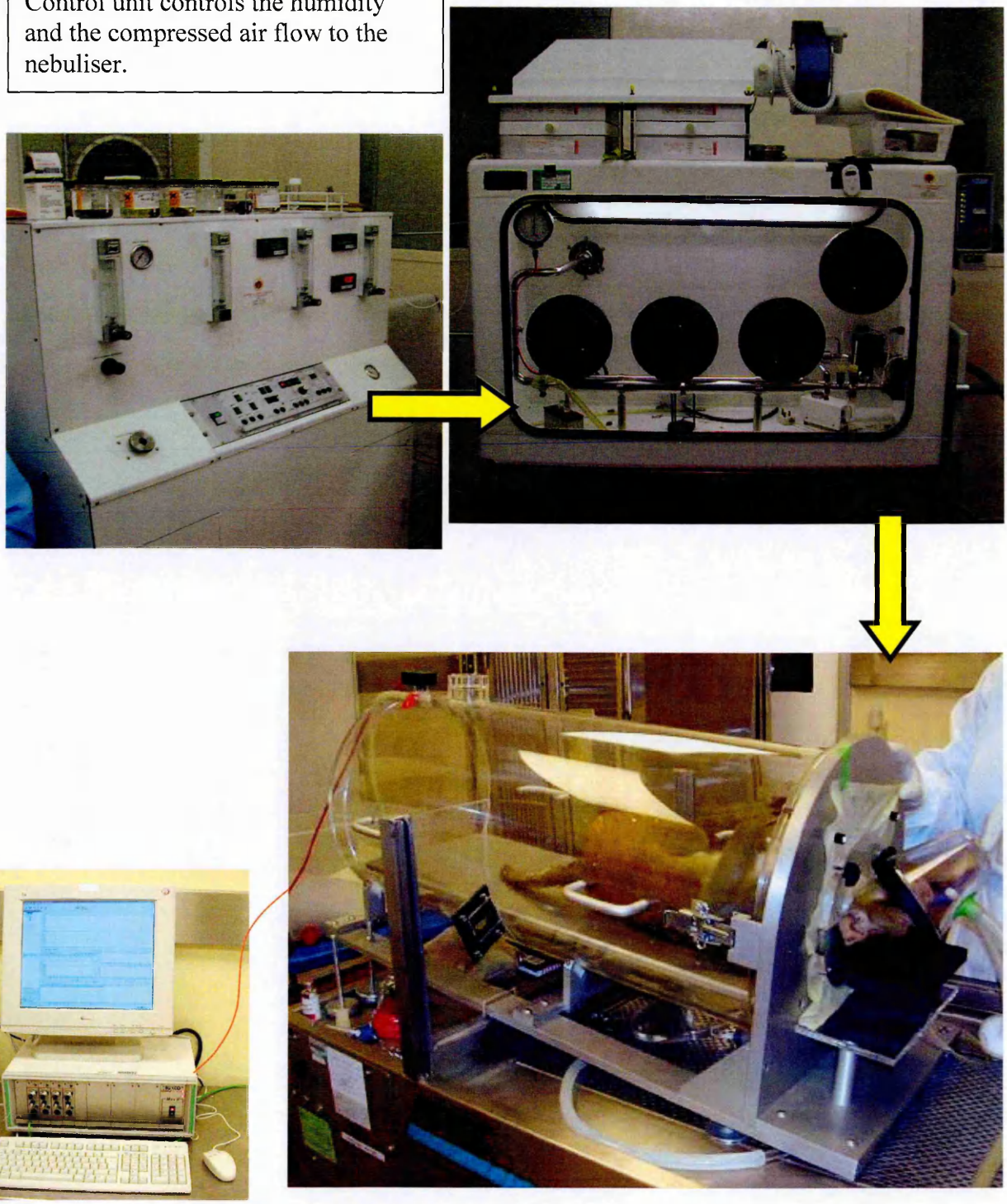
Recent reports looking at immune responses in rhesus macaques during the early course of infection have shown that IFN-γ-secreting cells are detected in PBMC by 2 months post-infection (Lewinsohn *et al.*, 2006), whereas in cynomolgus macaques responses are seen 2 weeks after infection (Lin *et al.*, 2006). However, the rhesus macaques were challenged with 1000 cfu of strain H37Rv whereas the cynomolgus macaques received 25 cfu of Erdman strain; both studies delivered the challenge strain by intratracheal instillation. Additionally, different antigens were used to stimulate the cells in the two studies, so the results are not directly comparable. To date, a time-course of IFN-γ

production during early TB disease has not been undertaken simultaneously in rhesus macaques and cynomolgus macaques.

For macaque experiments at the Centre for Emergency Preparedness and Response (CEPR), the dose that the animals received was calculated by the aerobiologist using values from the stock suspension used to generate the aerosol, the amount of bacilli circulating, and the volume of respiration of the animals. The apparatus used to challenge macaques is shown in figure 4.1.

Henderson equipment contained within a class III microbiological safety cabinet. *M. tuberculosis* suspension is aerosolised in a nebuliser and then circulated into the airflow.

Control unit controls the humidity and the compressed air flow to the nebuliser.



Macaques housed within an airtight chamber so that changes in air volume can be monitored. The computer provides information on lung volumes, respiration rates, and most importantly, amount of air inhaled. Aerosolised *M. tuberculosis* is given through an adapted mask.

Figure 4.1: Equipment used for challenging macaques with aerosolised *M. tuberculosis*.

In *M. tuberculosis*-infected macaques at CEPR, magnetic resonance imaging (MRI) was carried out on lungs removed at necropsy, in order to assess the distribution of the aerosol-delivered challenge dose. This approach has been carried out previously on guinea pigs; the results obtained indicated that lung lesions could be clearly visualised and that the application of three-dimensional stacking software could be utilised to give a reconstructed view of the whole-organ lesion distribution (Kraft *et al.*, 2004). These observations were repeated in the macaque lungs and demonstrated an extremely even distribution of lesions throughout both lungs (figure 4.2), thus supporting aerosol infection of macaques with *M. tuberculosis*.

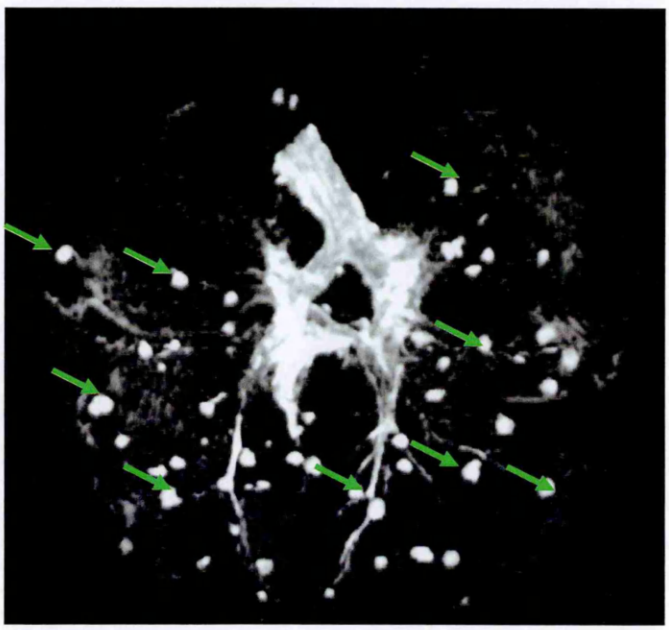


Figure 4.2: MRI image of lungs from a cynomolgus macaque 13 weeks after infection with aerosolised *M. tuberculosis*.

(Animal ID: 9909187. Visible granulomas are seen as white areas, examples shown with green arrows, spread throughout both lungs.)

4.1.3 Study aims

The main aims of the work described in this chapter are:

1) To determine IFN-γ responses post-challenge with aerosol-delivered *M. tuberculosis* in two non-human primate species (rhesus macaque and cynomolgus macaque).
2) To determine if there are any differences in IFN-γ dynamics between the two macaque species.
3) To assess whether the frequency of IFN-γ-secreting cells correlated with the amount of IFN-γ secreted.
4) To analyse whether IFN-γ measurements provide an indication of outcome or disease severity.

4.1.4 Chapter methods

In the challenge experiments, nine rhesus macaques and twelve cynomolgus macaques were infected with *M. tuberculosis*. The estimated mean doses given are shown in table 4.1.

	Estimated retained dose	
	Rhesus macaques	**Cynomolgus macaques**
1st challenge study	**30 cfu** (except D53, 500 cfu)	**30 cfu**
2nd challenge study	**40 cfu** (except D19, 70 cfu)	**40 cfu**
3rd challenge study	**45 cfu** (except D12, 75 cfu)	**40 cfu**
4th challenge study	-	**75 cfu**

Table 4.1: Challenge dose used in *M. tuberculosis* infection studies

Blood samples were taken from these animals before challenge to determine baseline levels of IFN-γ production, and then at 1, 2, 4, 6, 8, 10, and 12 weeks after challenge with *M. tuberculosis* (section 2.1.3.1).

The frequency of antigen-specific IFN-γ secreting cells was measured using the ELISPOT method (section 2.2.6) and the concentration of IFN-γ secreted after antigenic stimulation was measured using the ELISA method (section 2.2.7). Other cytokines were also measured in some of the *M. tuberculosis* infected animals using ELISA assays for IL-10 and IL-12 (section 2.2.7.2.2) and luminex analysis for TNF-α, IL-1β, IL-2 and IL-6 (section 2.2.7.3).

4.2 RESULTS

4.2.1 Enumeration of IFN-γ-secreting cells

4.2.1.1 Frequency of antigen-specific IFN-γ-secreting cells post *M. tuberculosis* challenge

Throughout the course of *M. tuberculosis* infection, the frequency of antigen-specific IFN-γ-secreting cells was monitored. During the 12-week study period post-challenge, four rhesus macaques (D12, D19, D28 and D53) were euthanised before experiment completion as they met humane clinical endpoints (e.g. 20% weight loss from maximum weight or showing signs of malaise). All cynomolgus macaques survived the duration of the study.

Following aerosol challenge with *M. tuberculosis*, the frequency of IFN-γ-secreting cells produced after *ex vivo* stimulation with PPD was measured in rhesus macaques (figure 4.3) and cynomolgus macaques (figure 4.4). As animals received different doses of *M. tuberculosis*, animals were grouped on the graphs according to their estimated dose. In both rhesus macaques and cynomolgus macaques, no apparent changes in the frequency of IFN-γ secreting cells could be related to differences in the dose of challenge. However, in the cynomolgus macaques challenged with a higher dose of 75 cfu, PPD-specific IFN-γ responses initially showed a peak at week 6 compared to week 4 in animals challenged with 40 cfu. As only PPD responses were measured at this time-point, it is not known whether responses to Ag85A, CFP10 or ESAT-6 peptide were also increased at this time.

Responses to all of the antigens (PPD and peptide pools to Ag85A, CFP10 and ESAT-6) were observed in the *M. tuberculosis*-challenged macaques during the 12 week study period with all animals making a response to at least one of the antigens tested. Increases in IFN-γ secreting cells were detected by 4 weeks post-infection, with few responses seen before this time-point.

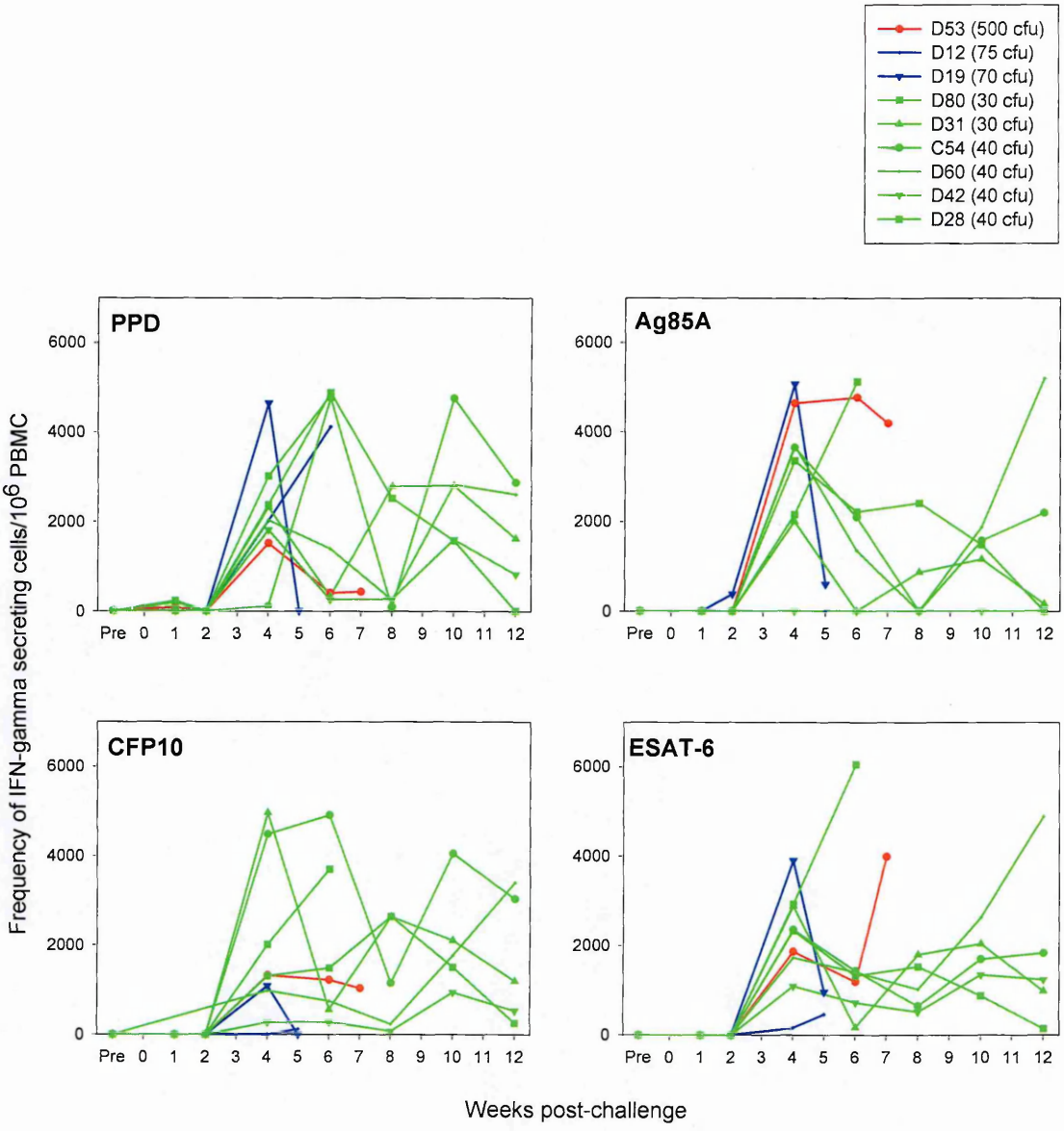


Figure 4.3: Frequency of antigen-specific IFN-γ-secreting cells following aerosol challenge with *M. tuberculosis* in rhesus macaques over the defined cut-off value.

(Cut-off defined as 3 times the mean value of pre-bleed counts. Coloured lines grouped according to estimated dose: red, >500 cfu; blue, >70 cfu; and green, <50 cfu).

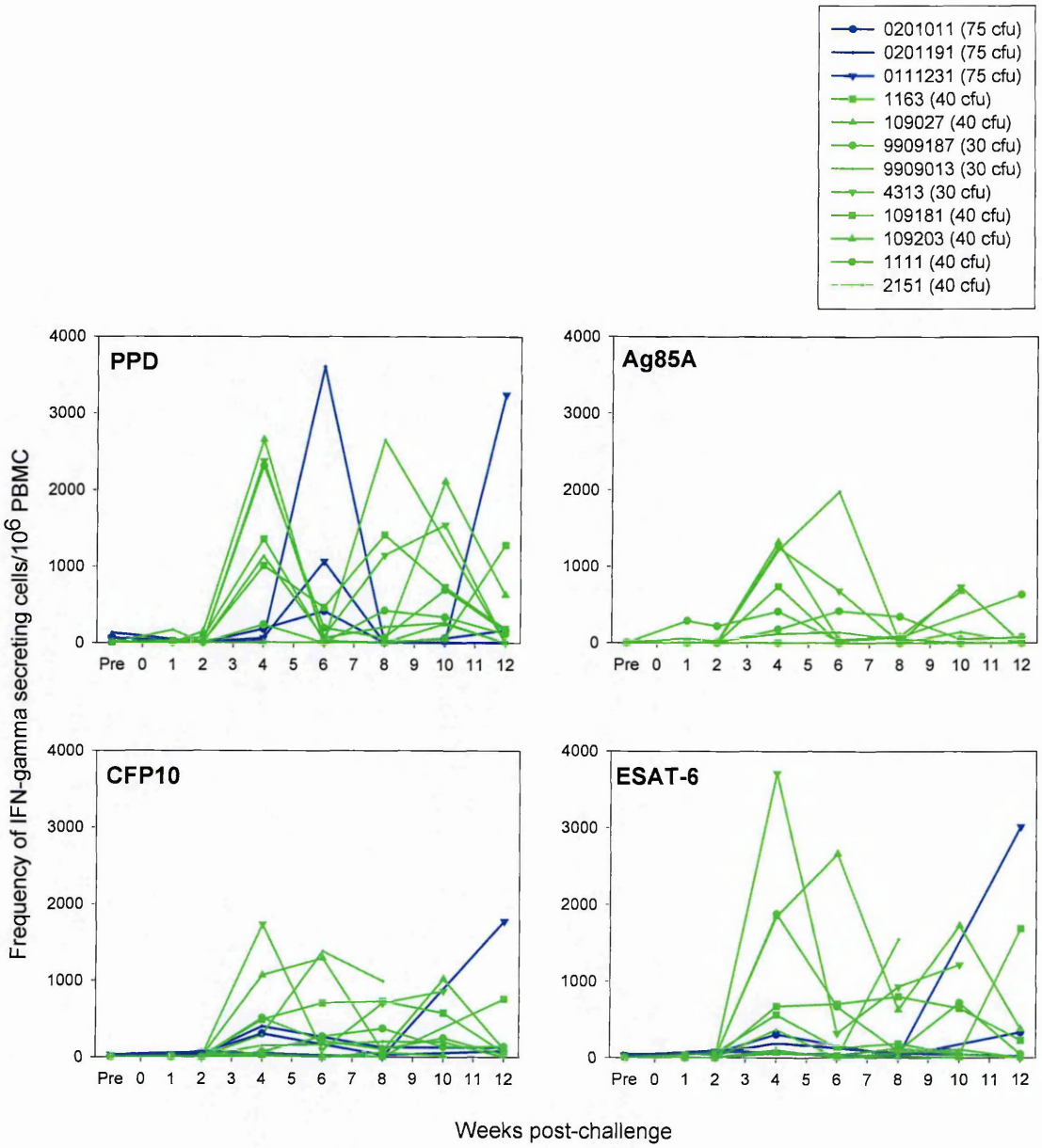


Figure 4.4: Frequency of antigen-specific IFN-γ-secreting cells following aerosol challenge with *M. tuberculosis* in cynomolgus macaques over the defined cut-off value. (Cut-off defined as 3 times the mean value of pre-bleed counts. Coloured lines grouped according to estimated dose: blue, >70 cfu; and green, <50 cfu).

4.2.1.2 Comparison of post-*M. tuberculosis* challenge responses between rhesus macaques and cynomolgus macaques

To determine whether there were any differences in frequencies of IFN-γ secreting cells after *M. tuberculosis* infection between rhesus macaques and cynomolgus macaques, a comparison was made between the two species (figure 4.5).

Using the Mann-Whitney statistical test, the differences in frequencies of IFN-γ-secreting cells between rhesus macaques and cynomolgus macaques were statistically significant ($P<0.05$) for PPD at 4, 6, and 10 weeks post-infection. For the peptide pools, significant differences were observed at 4, 6, 8 and 10 weeks post-infection. Results are summarised in table 4.2 and show that there are statistically significantly lower frequencies of antigen-specific IFN-γ-secreting cells in the cynomolgus macaques as compared to the rhesus macaques.

Mann-Whitney statistical p-value	**Weeks post *M. tuberculosis* infection**						
	1 week	**2 weeks**	**4 weeks**	**6 weeks**	**8 weeks**	**10 weeks**	**12 weeks**
PPD	0.6588	0.1098	0.0302	0.0049	0.0820	0.0046	0.0651
Ag85A	1.0000	0.2893	0.0081	0.0081	0.0164	0.0230	0.1709
CFP10	0.1939	0.7285	0.0116	0.0149	0.0234	0.0094	0.0234
ESAT-6	0.3538	0.1658	0.0077	0.0262	0.0234	0.0157	0.1425

Table 4.2: Statistical analysis comparing responses in rhesus macaques against those detected in cynomolgus macaques for the different antigen preparations tested. (Mann-Whitney test, green boxes show significant values where $P<0.05$).

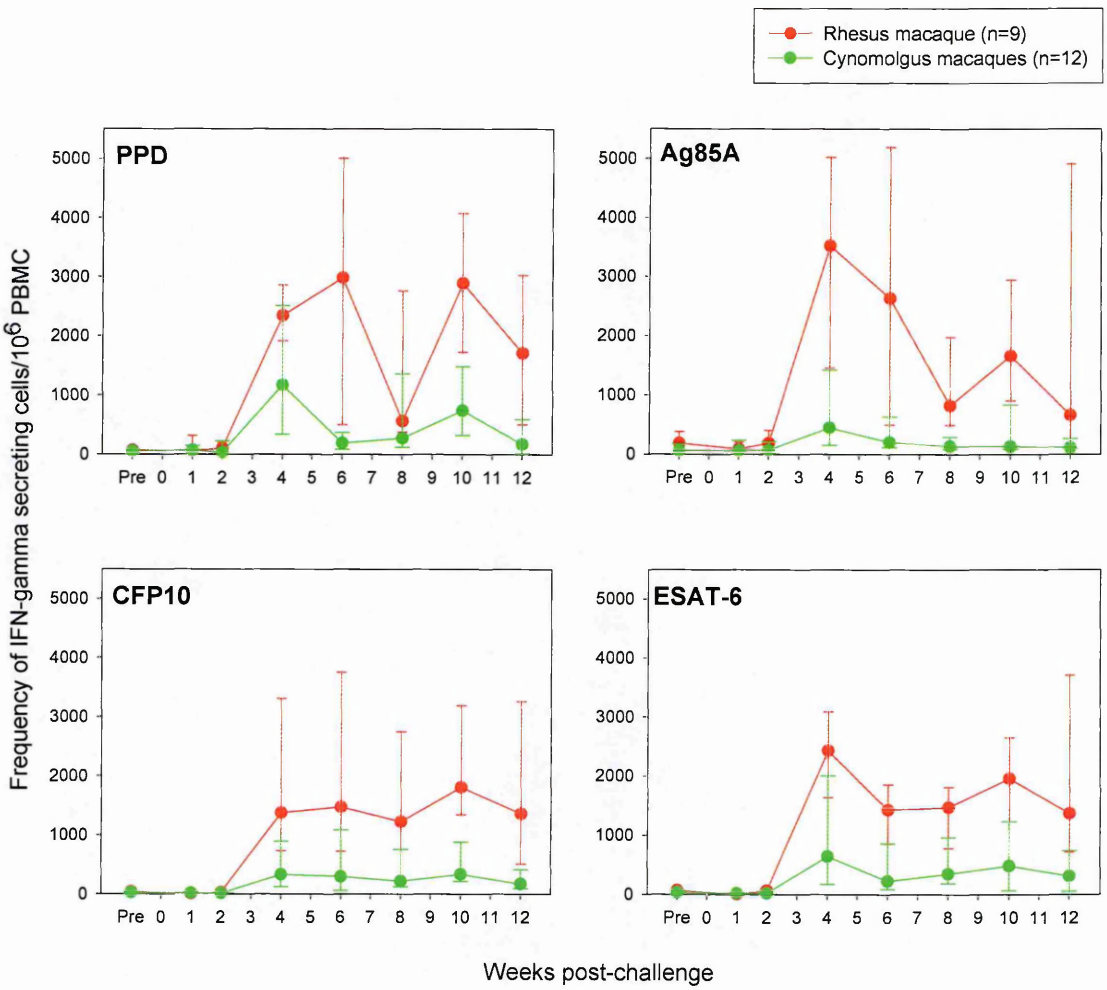


Figure 4.5: Comparison of median frequencies of IFN-γ secreting cells post-*M. tuberculosis* challenge in rhesus macaques and cynomolgus macaques.

(Red lines, rhesus macaques; green lines, cynomolgus macaques. Error bars denote interquartile range of samples).

4.2.1.3 Comparison between rapid progressors and 12 week survivors

From the nine rhesus macaques challenged with *M. tuberculosis*, four animals were terminated before the end of the planned 12 week study period. These animals were necropsied at week 5 (animal D19), week 6 (D28 and D12) and week 7 (D53) post-infection. As these animals met humane clinical endpoints it was clear that they had a more rapid progression to disease than those that survived the 12-week post-challenge study endpoint.

The frequency of IFN-γ-secreting cells between the 12-week survivors and the rapid progressors were plotted and compared (figure 4.6). Statistical analysis (table 4.3) showed that there were no significant differences between the frequencies of IFN-γ-secreting cells in animals that succumbed to rapid clinical disease and those that survived the 12 week study period.

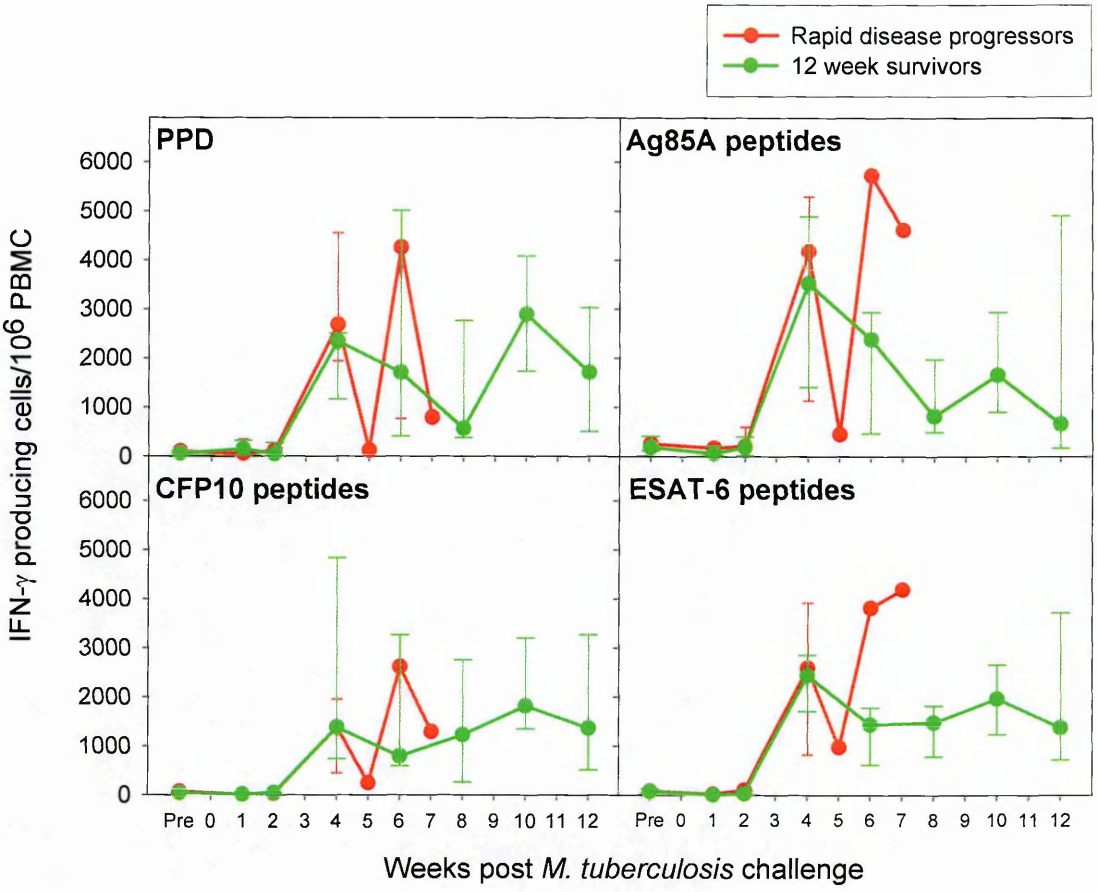


Figure 4.6: Comparison of median frequencies of IFN-γ-secreting cells in animals which survived the 12-week study period and those which were necropsied prior to the end of the study.

(12 week survivors, green lines; rapid progressors, red lines. Error bars denote interquartile range of samples).

Mann-Whitney statistical p-value	**Week 1**	**Week 2**	**Week 4**	**Week 6**
PPD	0.9025	1.0000	0.5403	0.8465
Ag85A	0.5403	*	0.7133	0.0814
CFP10	*	*	0.7133	0.5613
ESAT-6	*	0.5403	0.9025	0.5613

Table 4.3: Statistical test results comparing 12-week survivors against rapid disease progressors.

(Mann-Whitney test, significance confirmed if $P<0.05$; *, insufficient data to perform Mann-Whitney statistical test).

4.2.1.4 Responses to individual peptide pools post-*M. tuberculosis* challenge

Previous results for CFP10, ESAT-6 and Ag85A are shown as summed responses from multiple peptide pools for each of the antigens. For Ag85A the peptides are divided into 7 pools, and for CFP10 and ESAT-6 they are divided into 3 pools each.

The responses to each of the peptide pools of each of the antigens were plotted, in order to get an indication of whether any of the pools contained promiscuous epitopes recognised by several of the infected macaques (figures 4.7, 4.8 and 4.9). In addition, it may also indicate whether the immune response targets different parts of the antigen during the 12-13 week infection period.

CFP10 peptide pools 2 and 3 were dominant in both the rhesus macaques and cynomolgus macaques, with the exceptions of animals D12, 109181 and 1163 where peptide pool 1 gave the highest responses. For ESAT-6, pool 1 gave the highest frequencies of IFN-γ-secreting cells in 7 of the 9 rhesus macaques (78%) and pool 3 gave the largest responses in 6 of the 9 cynomolgus macaques (67%).

Unlike the CFP10 and ESAT-6 peptide pools, where sequential peptides are in each of the pools (e.g. peptides 1-5 are in pool 1, 6-10 in pool 2, and 11-15 in pool 3), peptides for Ag85A are dispersed non-sequentially (e.g. pool A contains peptides 1, 6, 15, 20, 29, 34, 43, 48, 57 and 64). This method was chosen to emulate the work carried out in human clinical trials, where epitope screening is more efficient if peptides are not sequential. Results show that pools A, D and E are the most immunogenic in the rhesus macaques and cynomolgus macaques. Interestingly, pool D of Ag85A induced a response in all of the animals except in one cynomolgus macaque (9909013).

Each animal displayed a similar profile of antigen-recognition throughout the duration of the experiment post-challenge. Where a peptide pool gave a high response, this continued throughout the period of study. Likewise, pools which gave low responses appeared to do so at each time point tested.

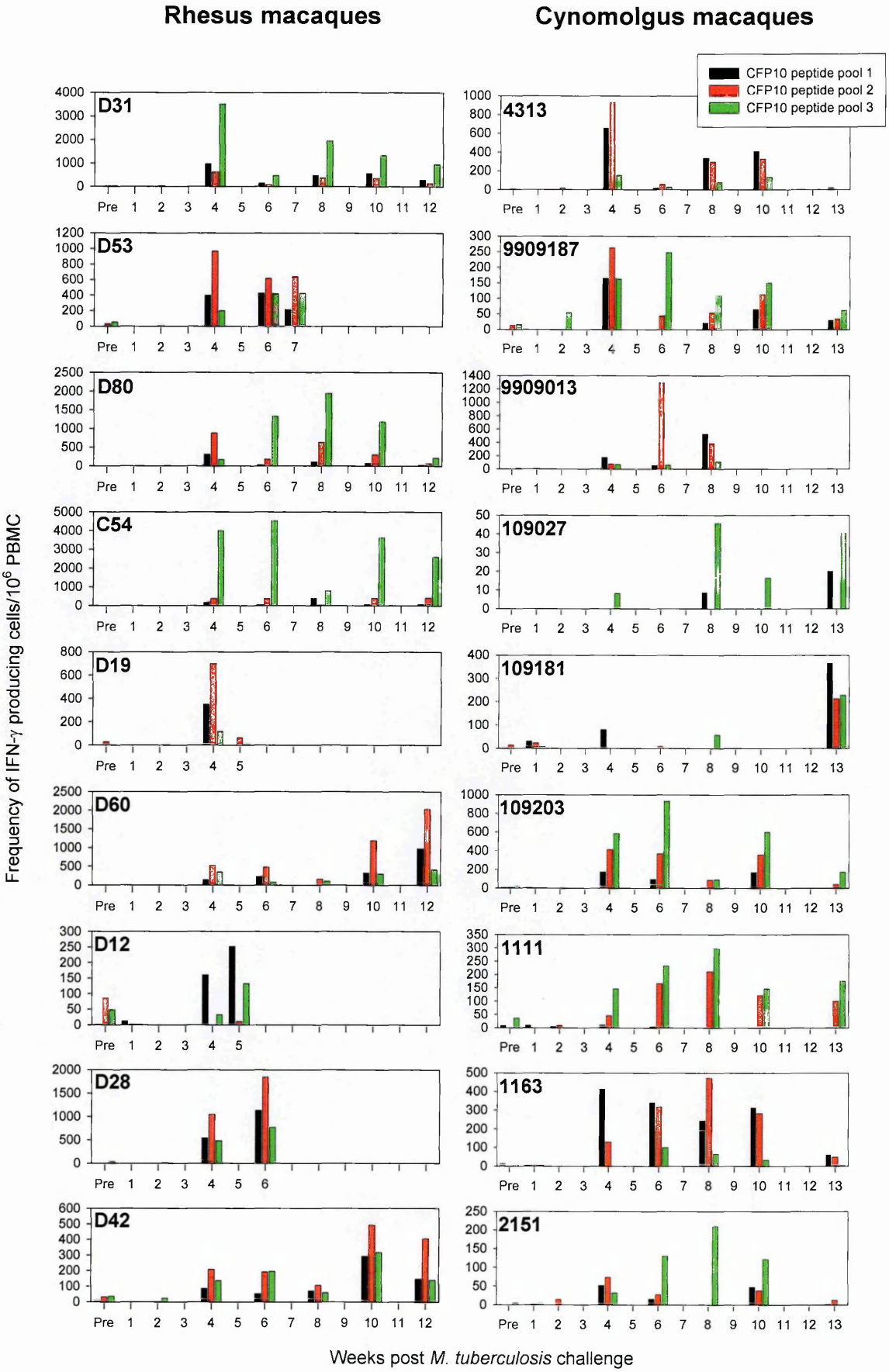


Figure 4.7: Responses by rhesus and cynomolgus macaques to CFP10 peptide pools post-challenge with *M. tuberculosis.*

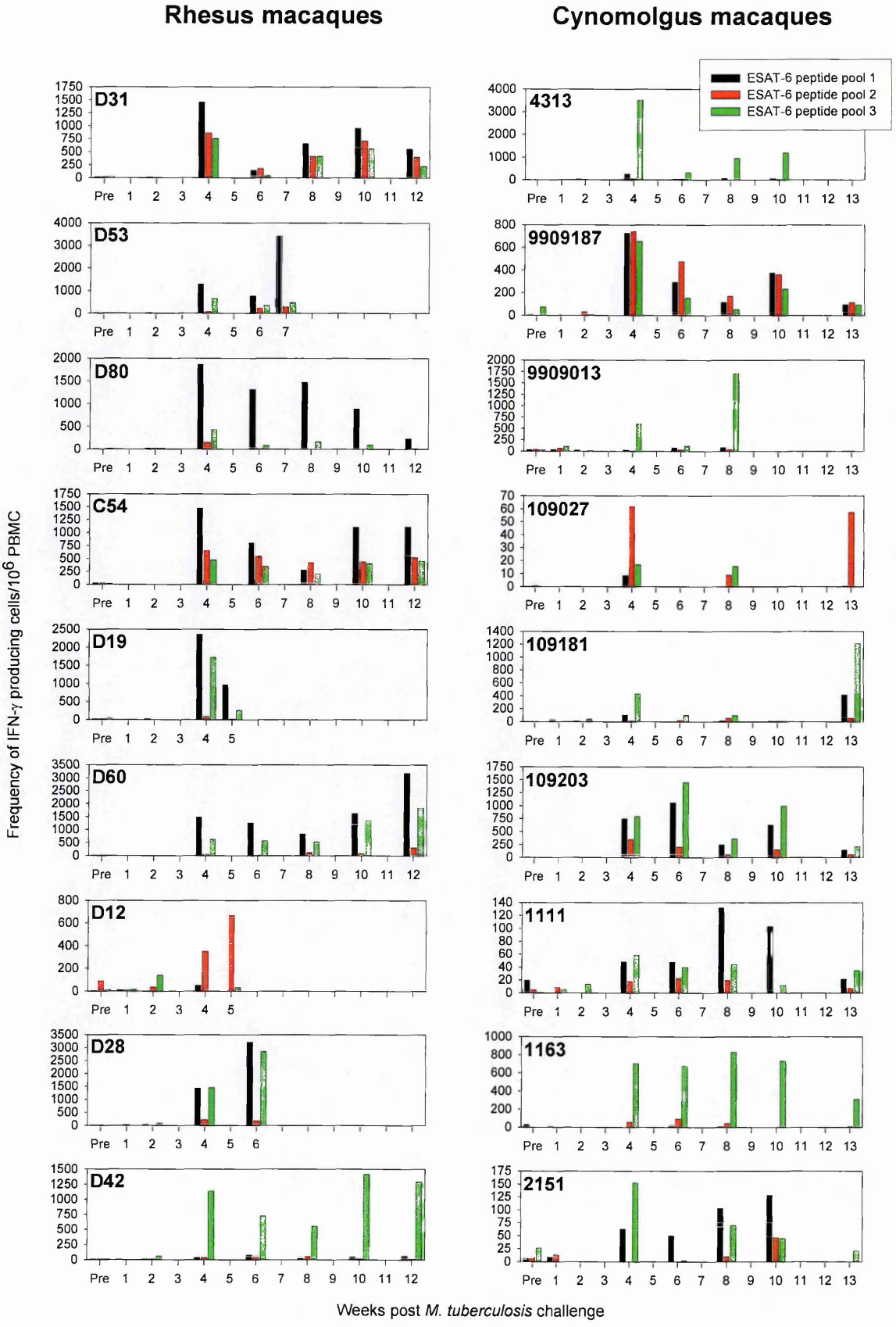


Figure 4.8: Responses by rhesus and cynomolgus macaques to ESAT-6 peptide pools post-challenge with *M. tuberculosis.*

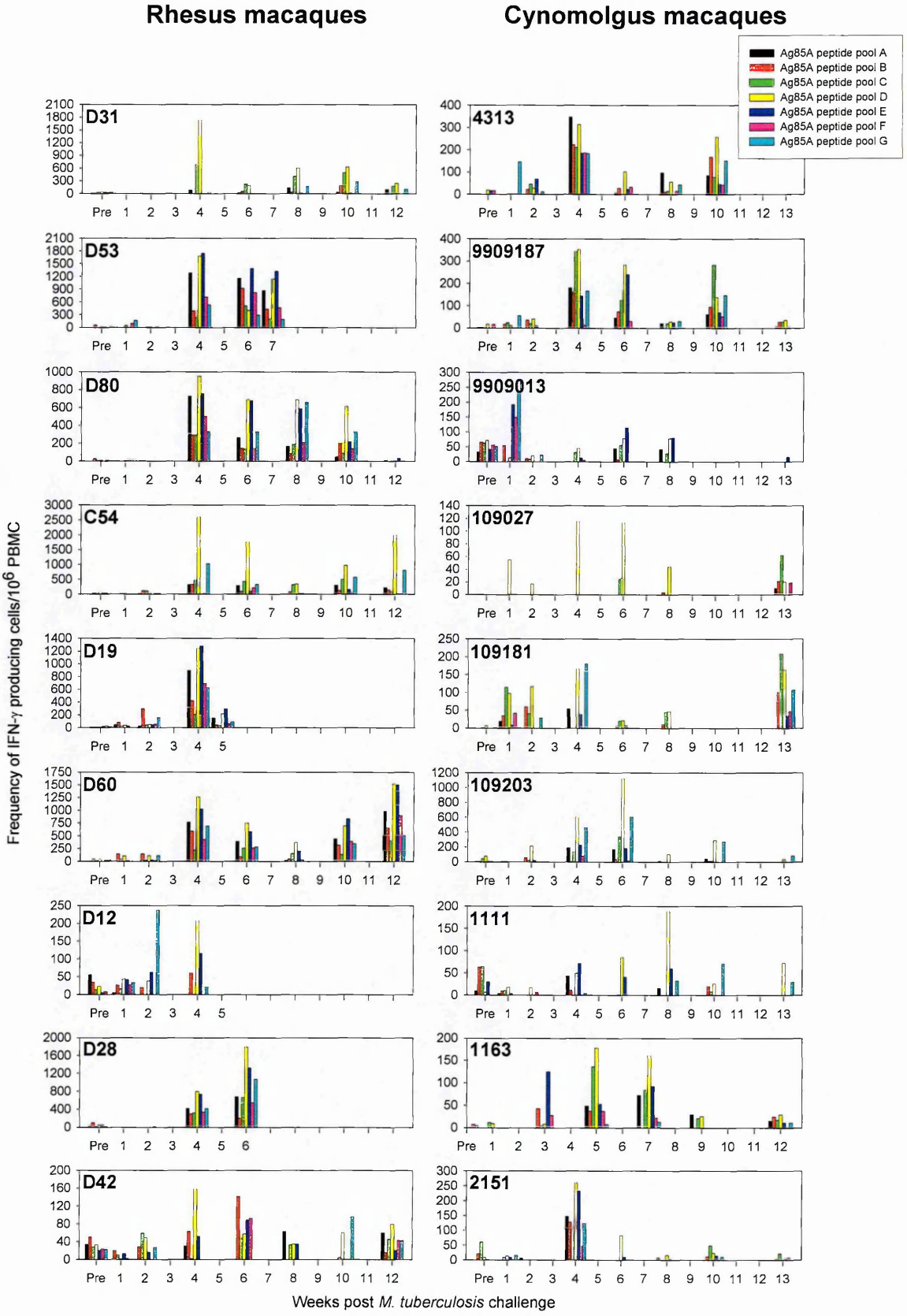


Figure 4.9: Responses by rhesus and cynomolgus macaques to Ag85A peptide pools post-challenge with *M. tuberculosis.*

4.2.1.5 Local IFN-γ responses in *M. tuberculosis*-challenged macaques

At the end of the experimental studies all animals were euthanised and necropsies performed, unless they showed advanced symptoms beforehand in which case they were terminated and necropsied for ethical reasons as soon as possible.

During post-mortem examination, samples were taken from the spleen, hilar lymph nodes and axillary lymph nodes for analysis of IFN-γ-secreting cells. Results from the rhesus macaques and cynomolgus macaques are shown in figures 4.10 and 4.11, respectively.

Rhesus macaques had similar frequencies of IFN-γ-secreting cells in the PBMC, spleen and hilar lymph node. Lower responses were detected in the axillary lymph nodes.

In the nine cynomolgus macaques the same observations were seen as with the rhesus macaques, with frequencies in the axillary lymph nodes being lower than that of the other tissues.

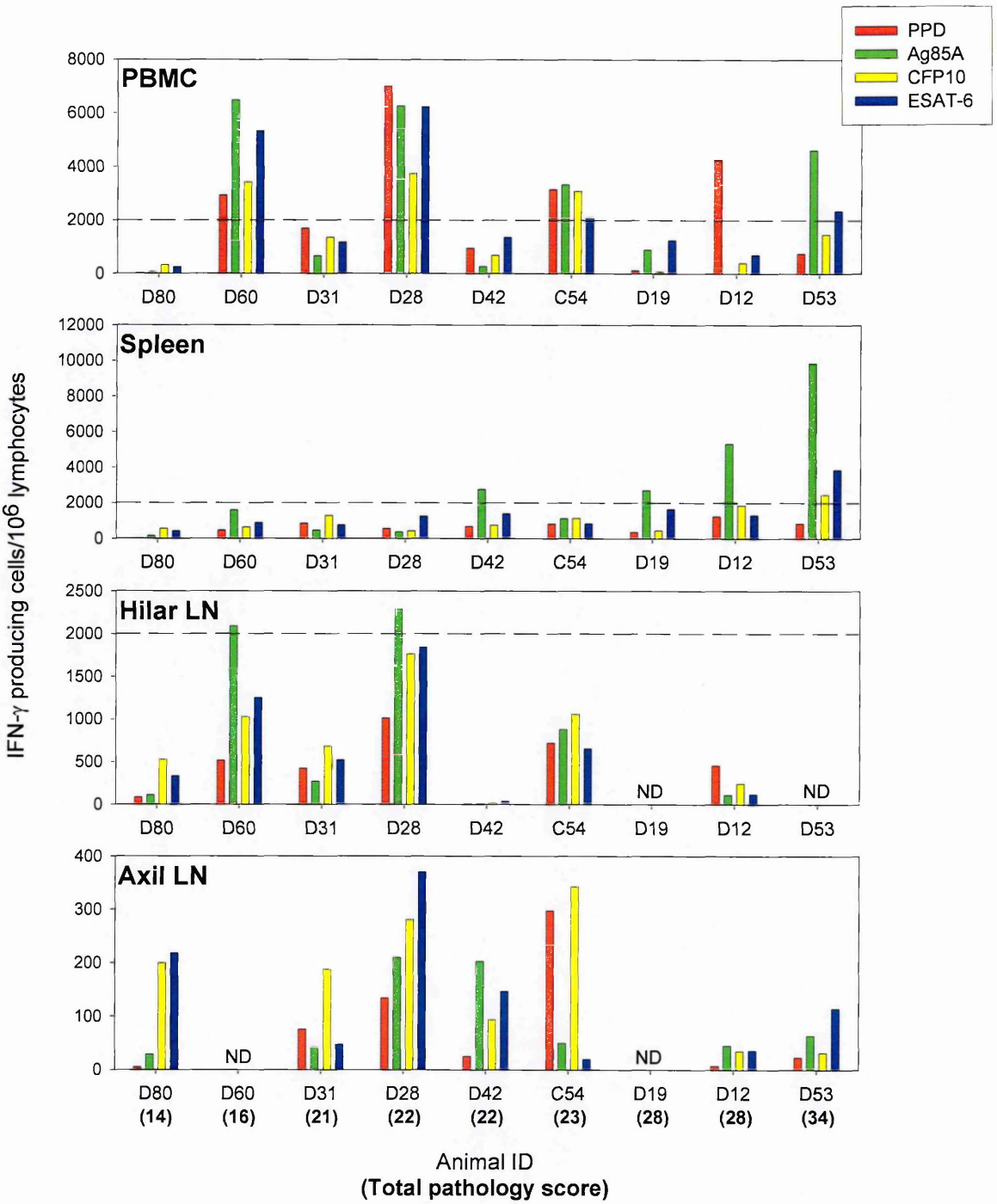


Figure 4.10: Frequencies of IFN-γ-secreting cells in local tissues taken at post-mortem of *M. tuberculosis* challenged rhesus macaques.

(Dashed line indicates a reference value of 2000 IFN-γ-secreting cells/10^6 PBMC; ND, not done).

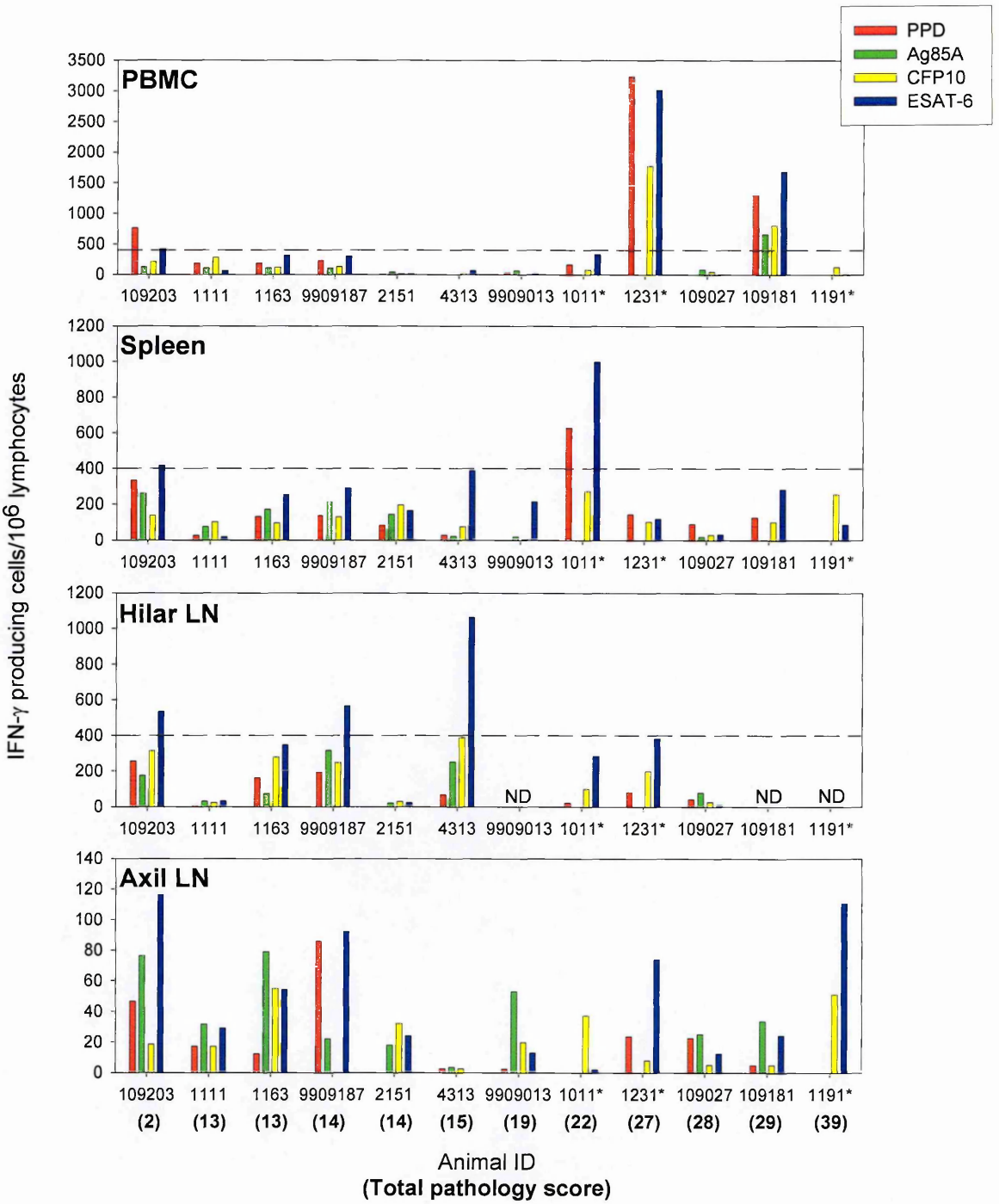


Figure 4.11: Frequencies of IFN-γ-secreting cells in local tissues taken at post-mortem of *M. tuberculosis* challenged cynomolgus macaques.

(Dashed line indicates a reference value of 400 IFN-γ-secreting cells/10^6 PBMC; ND, not done; *, Ag85A responses not measured).

Statistical correlation analyses was performed to determine whether local responses corresponded to severity of disease. The lesion:lung volume ratios were used to compare disease severity in the macaque models. This is a ratio of the volumes of the lesions compared to the total lung volume, and was measured by special software analysis of MRI scans (performed by Dr R. Basaraba). Data from 9 rhesus macaques and 9 cynomolgus macaques were compared, as lesion:lung volume ratios from animals in the 4^{th} challenge study (0111231, 0201011 and 0201191) were not available.

In rhesus macaques, higher frequencies of Ag85A- and ESAT-6-specific IFN-γ secreting cells in the spleen correlated with increased lesion:lung volume ratios (figure 4.12). In the cynomolgus macaques, however, no antigen-specific responses in the PBMC correlated with disease severity in the lungs (figure 4.13).

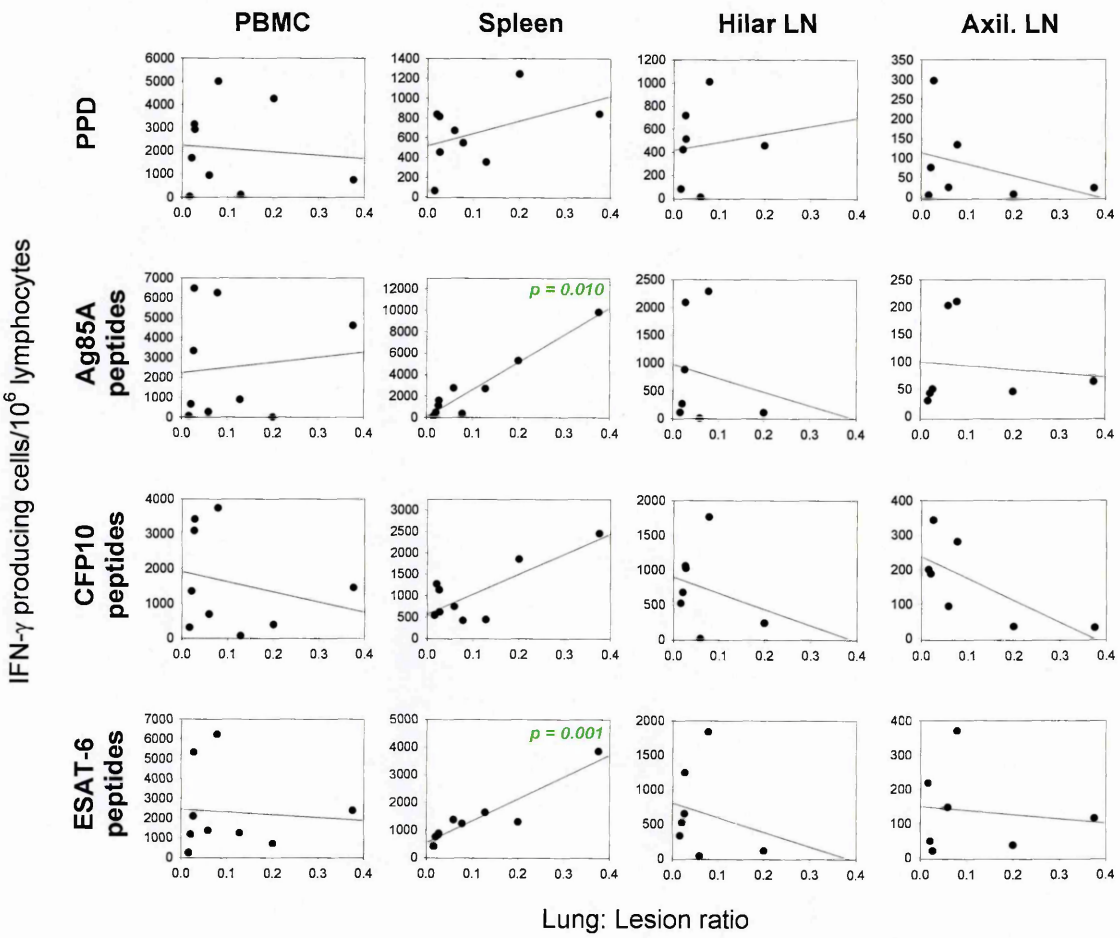


Figure 4.12: Comparison of disease severity with frequencies of IFN-γ-producing cells at local sites sampled at post-mortem in rhesus macaques.

(P-values represent significance levels of Spearman's rank correlation analysis).

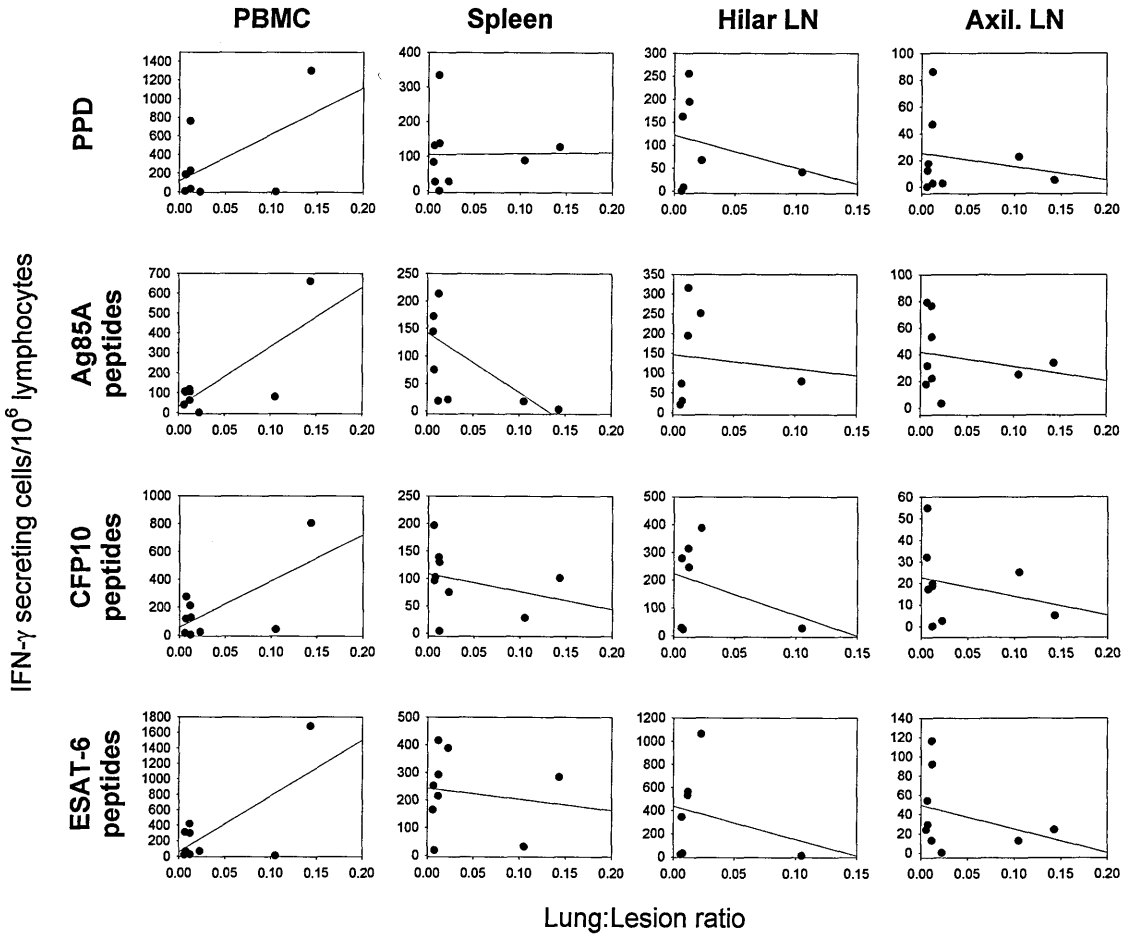


Figure 4.13: Comparison of disease severity with frequencies of IFN-γ-producing cells at local sites sampled at post-mortem in cynomolgus macaques.

(No values were significantly correlated using Spearman's rank correlation analysis).

4.2.1.6 Responding T-cell subsets of IFN-γ-secreting cells

At post-mortem, an increased volume of blood collection meant that more PBMC were available, thus allowing the depletion of $CD4^+$ and $CD8^+$ T-cell subsets to be carried out. By removing these subsets, their contribution to the IFN-γ response could be assessed. In each assay the same input number of cells were assayed in the ELISPOT experiments; therefore, data were not adjusted to take into account the relevant contribution of removed cells compared to unaltered PBMC preparations.

Results are shown as the percentage of response in the depleted populations compared to those detected in untreated PBMCs (figure 4.14). Responses were not measured in all of the *M. tuberculosis*-challenge animals, due to variations in cell yield and practical considerations. In most animals, the CD8-depleted preparations gave responses greater or equal to undepleted PBMC, indicating that removing the $CD8^+$ population did not contribute to the frequencies of IFN-γ-secreting cells. In contrast, when $CD4^+$ cells were removed, many of the responses declined thereby confirming that the primary cell subset producing IFN-γ at this time was $CD4^+$ T cells.

Although most animals showed mainly $CD4^+$ IFN-γ responses, there were two animals (rhesus macaque D19 and cynomolgus macaque 2151) that showed a bias towards $CD8^+$ IFN-γ production. This was to all of the peptides tested in both animals, although in animal 2151 only one peptide preparation was used. However, the PPD-induced IFN-γ responses did not show such bias towards $CD8^+$ cells.

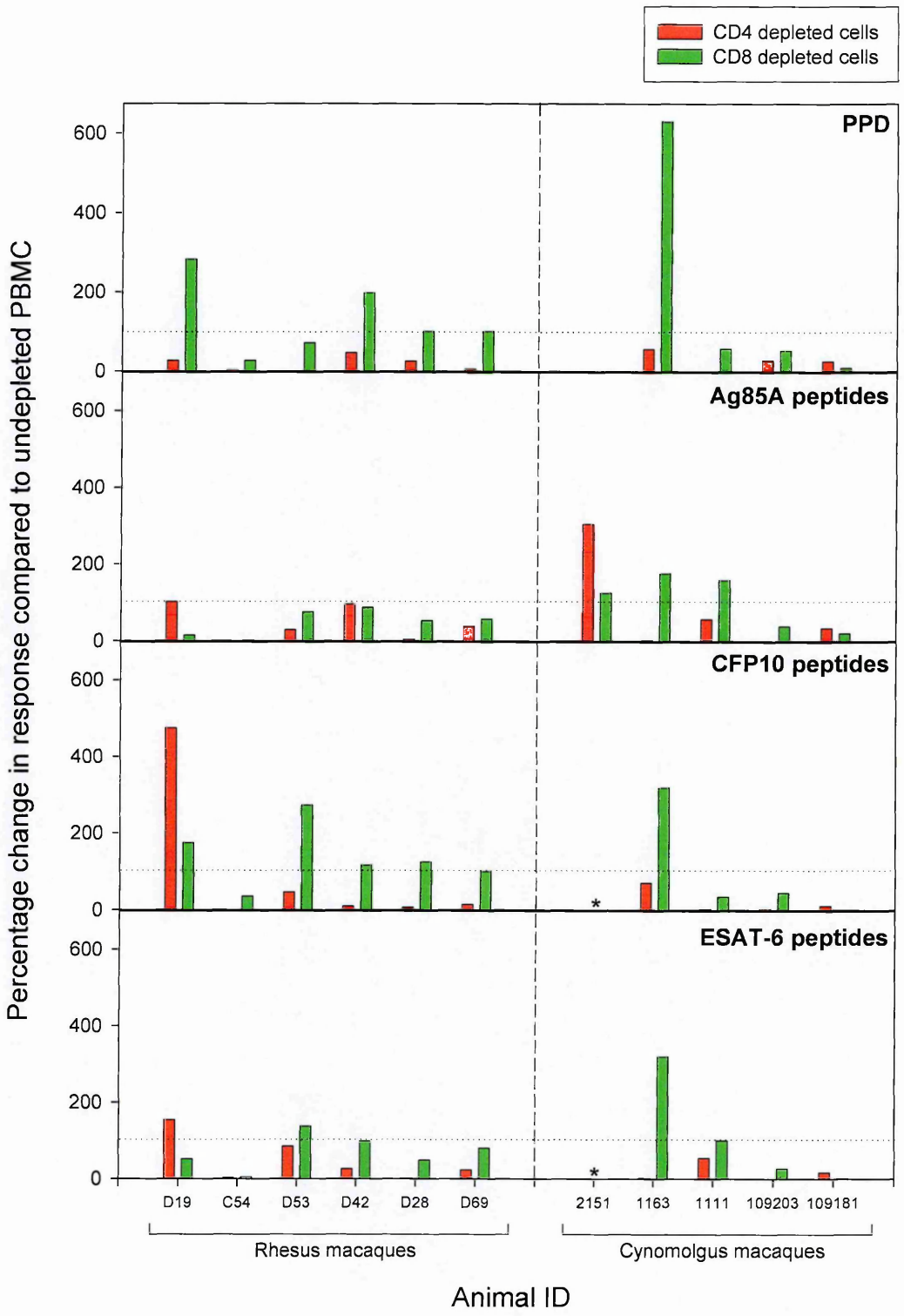


Figure 4.14: Effects of depleting $CD4^+$ and $CD8^+$ populations on IFN-γ cell frequencies in *M. tuberculosis* infected animals shown as percentage of response compared to levels seen in untreated PBMC.

(Dotted line represents a reference value of 100%; *, analysis not done).

4.2.1.7 Frequencies of IFN-γ-secreting cells across 12 weeks post-infection

When looking at responses post-challenge (figures 4.3 and 4.4), data from individual animals were displayed according to antigen stimulation. In some antigen-specific responses, a decline in response was observed at weeks 6-8. To investigate this further, responses from each individual animal that survived the 12-week post-challenge study period were plotted individually (figure 4.15), thus excluding the 4 rhesus macaques which met humane clinical endpoints mid-study.

Intriguingly, in the majority of both the rhesus and cynomolgus macaques there was a decline in response at weeks 6-8 post-challenge with *M. tuberculosis*. When there was a decline in response it was seen with all of the antigens tested, suggesting that this was a generic response not limited to certain antigen preparations.

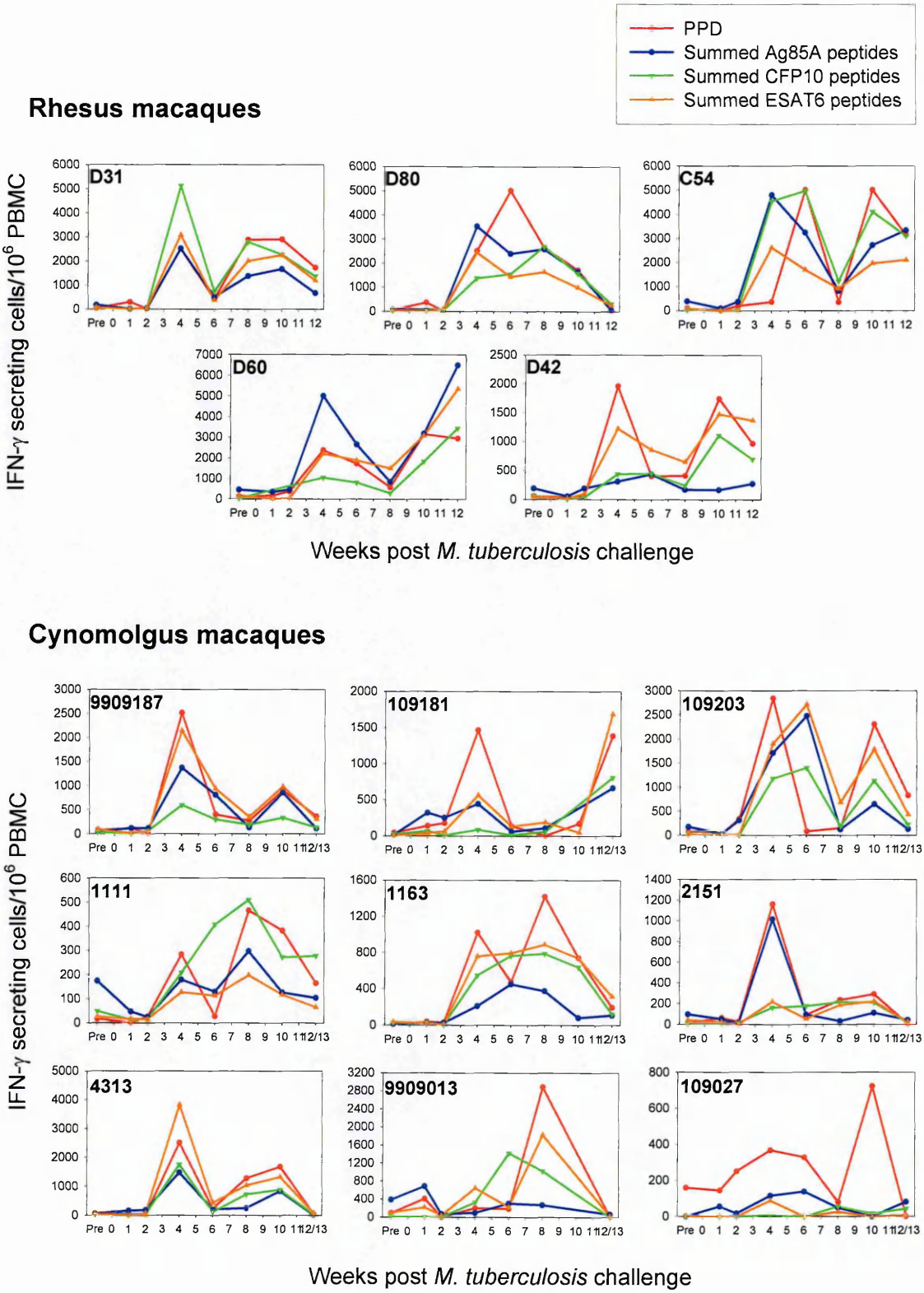


Figure 4.15: Results from individual macaques during the 12-weeks post-challenge with *M. tuberculosis* showing frequencies of IFN-γ-secreting cells to the antigens tested.

4.2.2 Concentrations of IFN-γ produced in peripheral cells from *M. tuberculosis*-challenged macaques

4.2.2.1 Responses after 6 days of diluted whole blood stimulation

In line with human studies (Black *et al.*, 2001; Black *et al.*, 2003; Black *et al.*, 2002), the amount of IFN-γ secreted after 1:10 diluted whole blood had been stimulated for 6 days with PPD or BCG was measured. This assay was carried out in samples taken from animals in the first challenge study. Results for both antigens are shown in figure 4.16, with all animals showing responses above the cut-off value of 100 pg/ml for this assay.

IFN-γ was first detected in the supernatants 6 weeks after challenge with *M. tuberculosis.* The amount of IFN-γ secreted in the rhesus macaques was similar with both antigens tested: PPD and BCG. However, in the cynomolgus macaques, 2 of the animals secreted more IFN-γ in response to simulation with PPD than with BCG.

There was a significant correlation in the IFN-γ concentrations secreted after stimulation with PPD and BCG (Spearman's rank correlation: rhesus macaques, r_s=0.842, P<0.001; cynomolgus macaques, r_s=0.859, P<0.001). As both antigens gave comparable profiles, and PPD is used for stimulation in the ELISPOT assay, for future experiments only PPD responses were monitored in the whole blood ELISA assay.

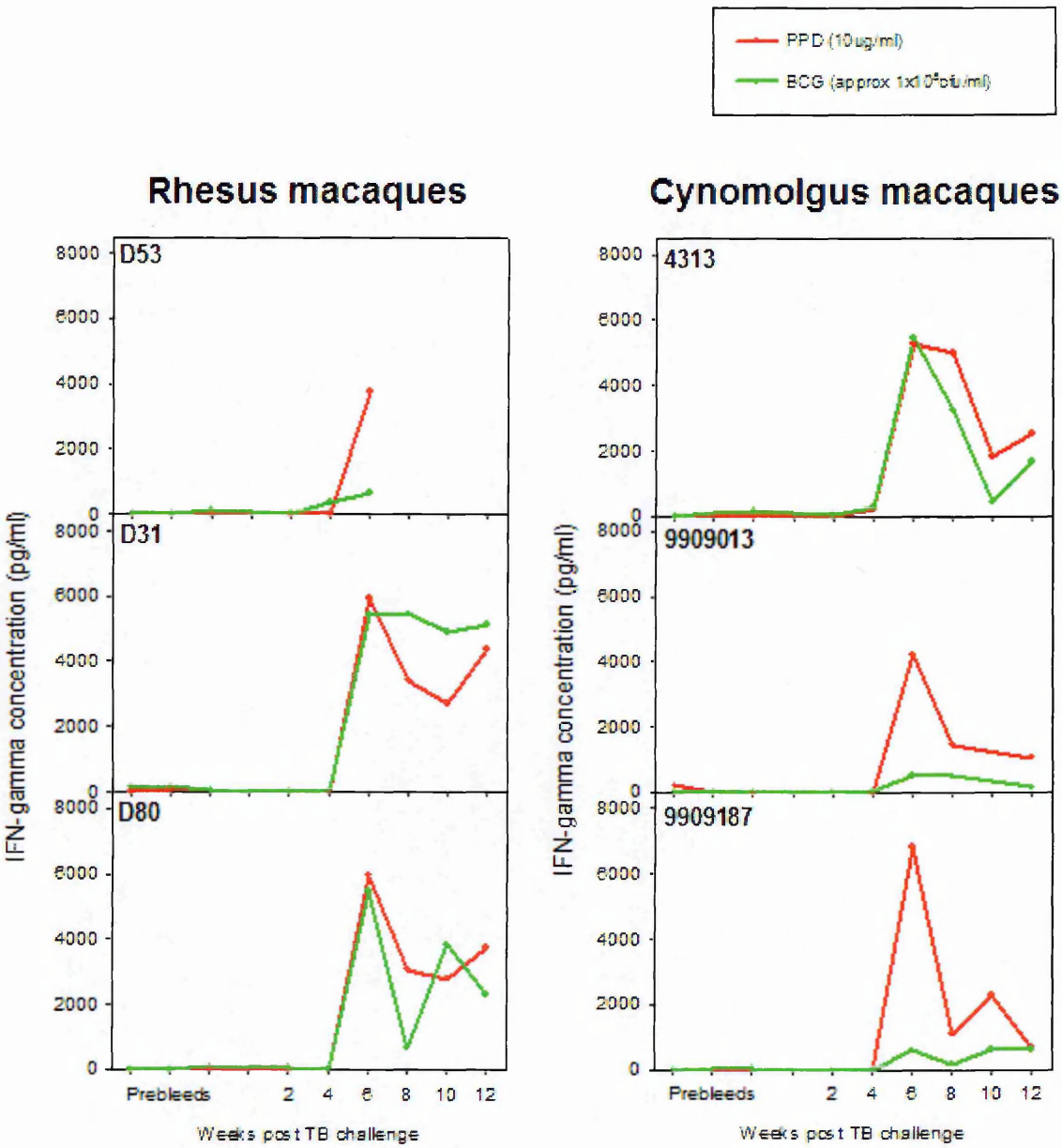


Figure 4.16: Concentrations of IFN-γ secreted after 6 days incubation of diluted whole blood in rhesus macaques and cynomolgus macaques challenged with *M. tuberculosis.* (PPD-stimulated, red line; BCG-stimulated, green line).

As PPD antigen was used to measure both the concentration of IFN-γ secreted and the frequency of IFN-γ-secreting cells, a comparison was made between the two assays. Results are shown in figure 4.17. The data show that the two responses are not comparable, as the peaks in the frequency of IFN-γ-secreting cells do not often occur at the same times as the peaks in the concentration of IFN-γ secreted. Statistical analysis confirmed that the two assays were not correlated (Spearman's rank correlation coefficient: rhesus macaques, r_s=0.375, P=0.125; cynomolgus macaques, r_s=0.372, P=0.106).

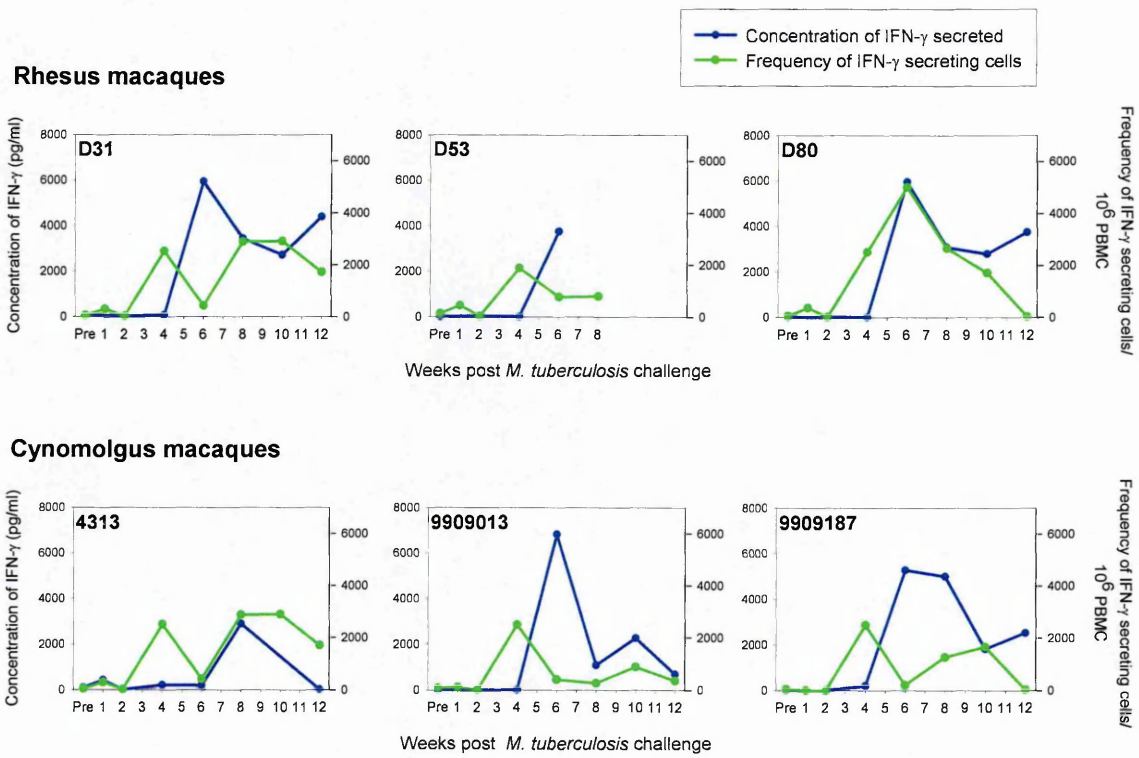


Figure 4.17: Comparison between the frequency of PPD-specific IFN-γ-secreting and the amount of IFN-γ secreted in diluted blood after 6 days of stimulation with PPD. (Frequency of cells, blue line; concentration secreted, green line).

4.2.2.2 Responses after 24 hours of diluted whole blood stimulation

To determine whether the differences in response seen between the frequency of IFN-γ-secreting cells and the amount of secreted IFN-γ in 6 day whole blood supernatants was due to the length of stimulation, a different approach was taken with a group of *M. tuberculosis*-challenged macaques. As the increased stimulation of 6 days in the whole blood assay may allow an amplification of responding cells, or the ability for more cell subsets to contribute to IFN-γ secretion, the time of stimulation in both assays was made identical. Diluted whole blood was stimulated overnight, in parallel with the IFN-γ ELISPOT assay, and concentrations of IFN-γ in the supernatants assessed by ELISA.

Results from these experiments are shown in figure 4.18. Responses in both assays start being detected at 4 weeks post-challenge with *M. tuberculosis*. It was noticed that there were comparable trends in the data, and this was confirmed by the data being statistically correlated (Spearman's rank correlation coefficient: rhesus macaques, r_s=0.764, P=0.001; cynomolgus macaques, r_s=0.831, P<0.001). However, the sample size (n=3 from each macaque species) remains small.

Therefore, results between the ELISPOT and whole blood ELISA assay are comparable when both are incubated overnight.

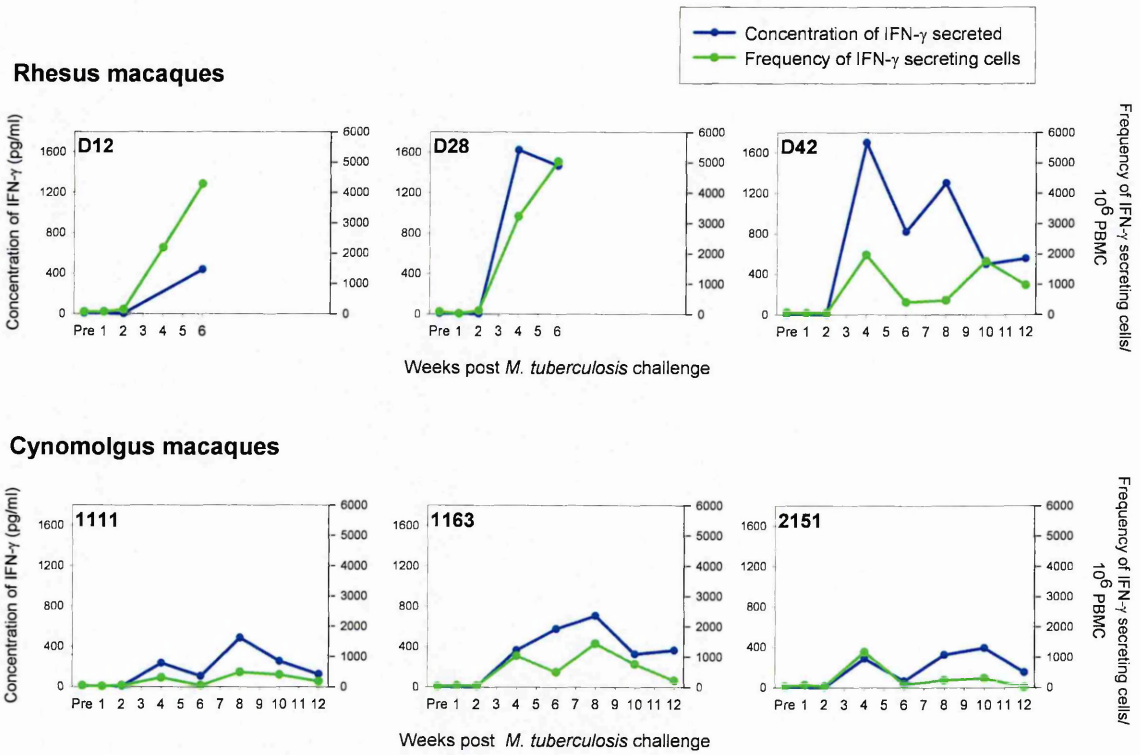


Figure 4.18: Comparison between the frequency of PPD-specific IFN-γ-secreting cells and the amount of IFN-γ secreted in diluted blood after overnight stimulation with PPD. (Frequency of cells, blue line; concentration secreted, green line).

4.2.2.3 Responses after 3 days of PBMC stimulation

To align data with other non-human primate studies where this analysis has been carried out in rhesus macaques and cynomolgus macaques (Langermans *et al.*, 2001; Langermans *et al.*, 2005), and to stimulate a known number of cells, PBMC were used instead of whole blood. Additionally, the period of stimulation was 3 days.

Concentrations of IFN-γ secreted in the 3 day PBMC stimulation assay were compared with the frequencies of IFN-γ-secreting cells (see figure 4.19). Although there were some time-points where a high concentration of IFN-γ was secreted with a high frequency of IFN-γ-secreting cells, generally, results between the two assays did not produce high levels of correlation (Spearman's rank correlation: rhesus macaques, r_s=0.750; cynomolgus macques, r_s=0.458).

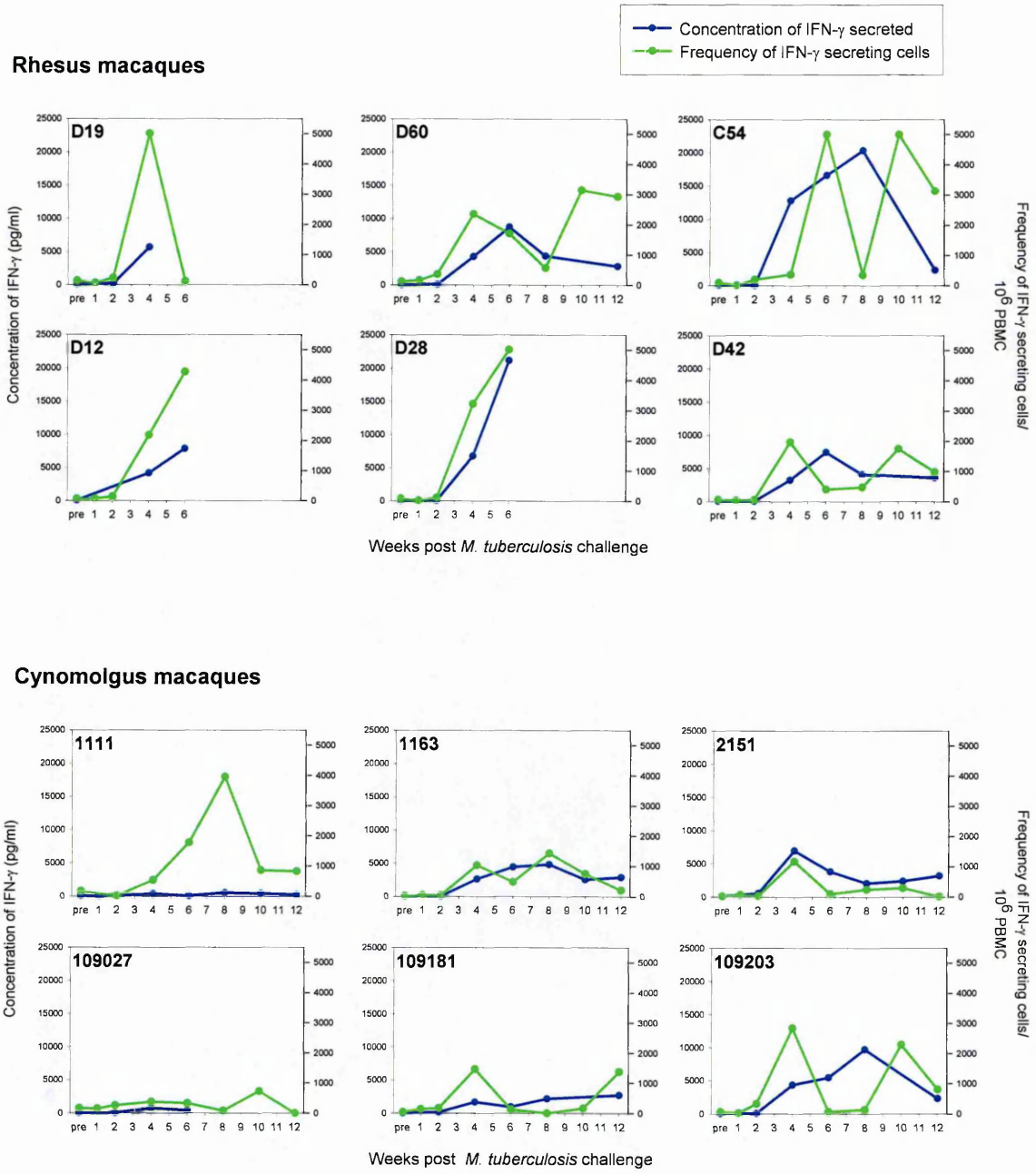


Figure 4.19: Comparison between the frequency of PPD-specific IFN-γ-secreting cells and the amount of IFN-γ secreted in supernatants from PBMC stimulation with PPD for 3 days.

(Frequency of cells, blue lines; concentration secreted, green lines).

The levels of IFN-γ secreted in PBMC stimulated for 3 days were used in two of the challenge studies, thereby providing data from six rhesus macaques and six cynomolgus macaques. The amount of IFN-γ secreted into the supernatants of these animals is shown in figure 4.20.

Secretion of IFN-γ was detected 4 weeks post-challenge with *M. tuberculosis* in both the rhesus macaques and the cynomolgus macaques. Two rhesus macaques (D19 and D12) were challenged with >50 cfu *M. tuberculosis*. However, no apparent difference in the concentration of IFN-γ secreted was observed with these higher doses. Apart from two rhesus macaques (C54 and D60) secreting higher levels of IFN-γ (>10,000 pg/ml), the other rhesus macaques secreted concentrations similar to those detected in the cynomolgus macaques (≤10,000 pg/ml).

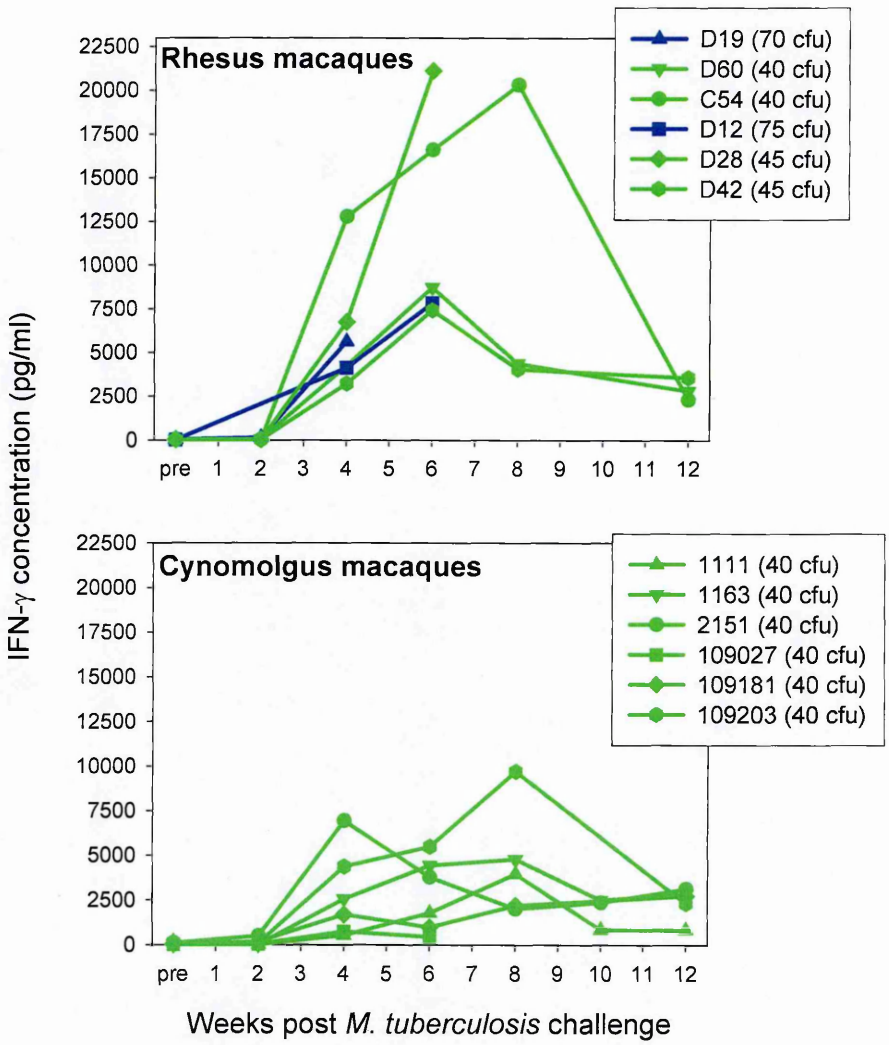


Figure 4.20: Concentrations of IFN-γ secreted after 3 days incubation of PBMC with PPD in animals challenged with *M. tuberculosis.*

(Challenge dose >50 cfu, blue lines; <50 cfu, green lines).

Three of the rhesus macaques (D19, D12 and D28) studied had to be euthanised before the 12-week follow-up period due to rapid progression of *M. tuberculosis* infection. To determine whether these animals showed differences in concentrations of IFN-γ secreted they were compared with 12-week survivors (figure 4.21). These results show that there were no differences in IFN-γ secretion between animals which rapidly progressed to disease and those which survived until 12 weeks post-*M. tuberculosis* infection.

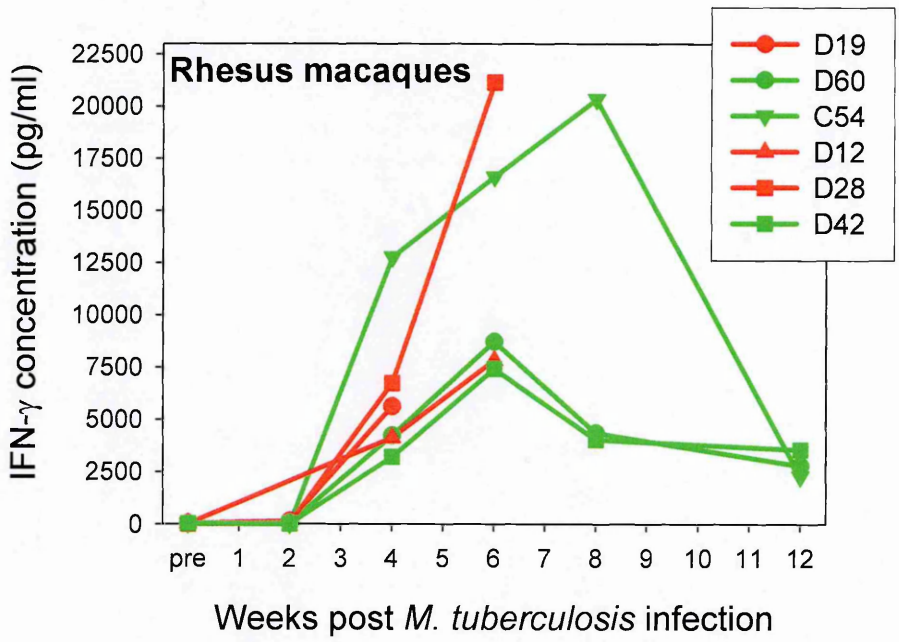


Figure 4.21: Comparison of IFN-γ secreted in 3 day, PPD-stimulated PBMC supernatants between animals with rapid disease progression and 12-week survivors. (Rapid progressors, red lines; 12-week survivors, green lines).

4.2.2.4 Comparison of IFN-γ secretion in rhesus macaques and cynomolgus macaques

Different conditions (e.g. incubation times and sample preparations) were tested, so differences between concentrations of IFN-γ secreted in rhesus macaques and cynomolgus macaques were compared for all experiments.

Results show a difference in the 3 day PBMC supernatants 6 weeks post-challenge between the two species, but at all the other time-points and in all of the other sample preparations no statistically significant differences were observed (table 4.4).

Therefore, after challenge with *M. tuberculosis* rhesus macaques and cynomolgus macaques produce similar amounts of IFN-γ after stimulation with PPD. This is in direct contrast to the differences observed in the frequency of IFN-γ-secreting cells, where cynomolgus macaques had significantly fewer antigen-specific IFN-γ-producing cells compared to rhesus macaques (shown previously in table 4.2).

Mann-Whitney statistical p-value	Weeks post *M. tuberculosis* challenge					
	2	4	6	8	10	12
24hr whole blood	*	0.1489	0.1904	0.0809	0.0809	0.0809
3 day PBMC	0.3153	0.1282	0.0081	0.3711	*	0.7656
6 day whole blood	0.6625	1.0000	1.0000	0.7728	0.2453	0.1489

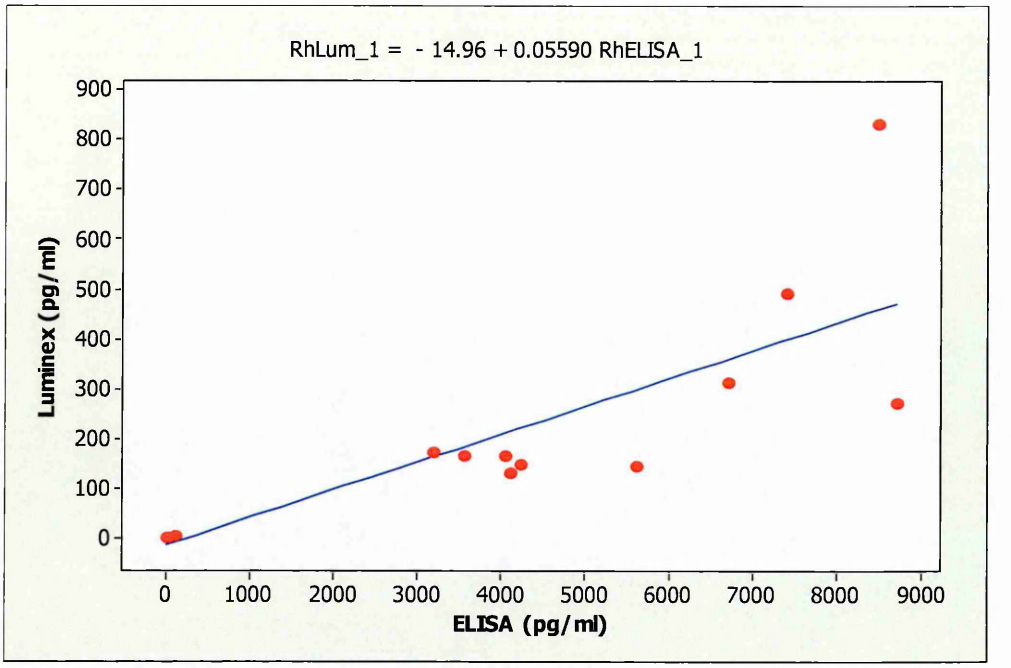
Table 4.4: Statistical significance values when IFN-γ secreted from rhesus macaques and cynomolgus macaques is compared in the different experimental conditions.
(Mann-Whitney statistical test, significant level is $P<0.05$; *, insufficient data to perform statistical analysis).

4.2.2.5 Detection of multiple cytokines using luminex analysis

Supernatants from the 3 day PBMC stimulation were used in a multiplex assay (luminex) to detect multiple cytokines simultaneously in the same sample. In addition to IFN-γ, four other cytokines were tested: TNF-α, IL-1β, IL-2 and IL-6. Due to their importance in contributing to the kinetics of IFN-γ production, IL-10 and IL-12 were also monitored using an ELISA assay, as luminex reagents specific for non-human primates were not available for these cytokines.

To ensure that the luminex assay gave consistent results, IFN-γ results were compared with those obtained by ELISA. Results are shown in figure 4.22. These show that both assays were significantly correlated (Spearman's rank correlation coefficient: rhesus macaques, r_s=0.900, P<0.001; cynomolgus macaques, r_s=0.961, P<0.001). However, equations from the fitted lines show that luminex concentrations are approximately 20 times less than those of the ELISA assay. Therefore, although trends are correlated, the actual concentrations differ.

Rhesus macaques:



Cynomolgus macaques:

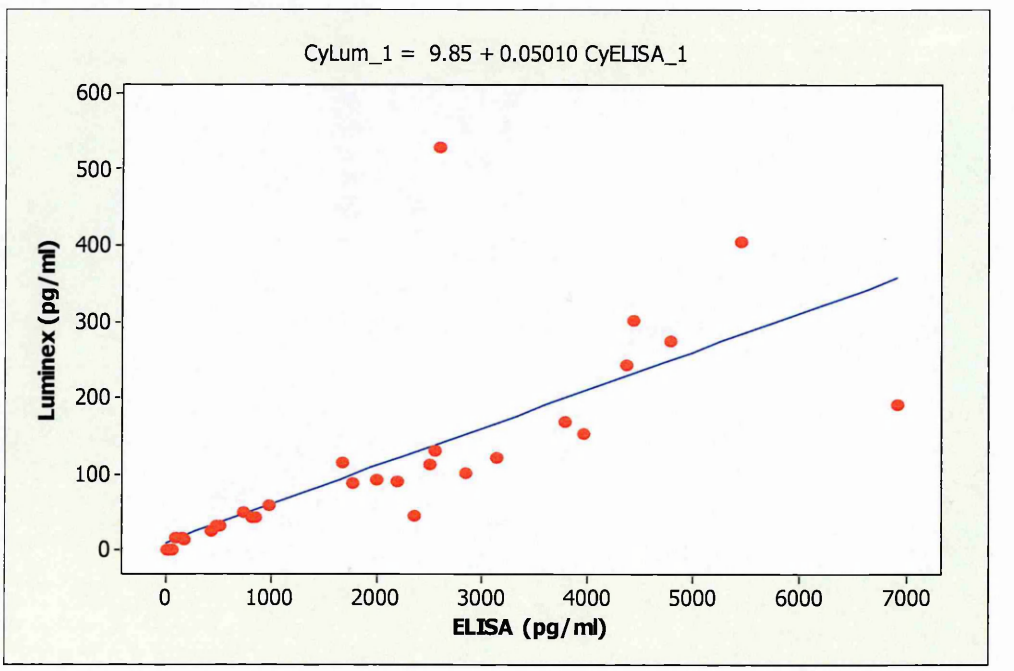


Figure 4.22: Comparison of IFN-γ concentrations in the ELISA and luminex assays using 3 day PBMC supernatants post *M. tuberculosis*-challenge.

Secretion profiles of TNF-α, IL-1β, IL-2, IL-6, IL-10 and IL-12 in 3 day, PPD-stimulated PBMC supernatants are shown in figure 4.23.

IL-10 and IL-12 were quantified by ELISA assays. IL-10 was detected in one rhesus macaque (C54; 12 weeks post-challenge), and in two cynomolgus macaques (1111 and 2151; 8 and 6 weeks post-challenge, respectively). In the remaining animals it was either not detected or gave results below baseline values. Concentrations of IL-12 never exceeded those observed prior to infection. However, the results show that the responses prior to challenge were subsequently diminished during *M. tuberculosis* infection.

The remaining cytokines were quantified using luminex analysis. Secretion of TNF-α was observed in all three rhesus macaques and all five cynomolgus macaques, with peak production varying but generally being at around weeks 6-8 post-infection. IL-2 production was seen 6-8 weeks after infection with *M. tuberculosis* in most of the challenged macques. However, IL-2 was not produced in rhesus macaque D60 or cynomolgus macaque 109181. IL-6 was detected in all of the rhesus macaques, from week 4 post-infection in D42 but 8 weeks after challenge in animals C54 and D60. In the cynomolgus macaques, only two of the five animals (40%) responded at weeks 4 (1163) and 8 (109203). The other three animals had levels similar to those observed prior to infection. Detection of IL-1β was seen in all three rhesus macaques studied, with levels peaking at weeks 6 (D42) and 8 (C54 and D60). In the cynomolgus macaques only animals 1163 and 109203 produced IL-1β which was also detected first at weeks 6 and 8, respectively.

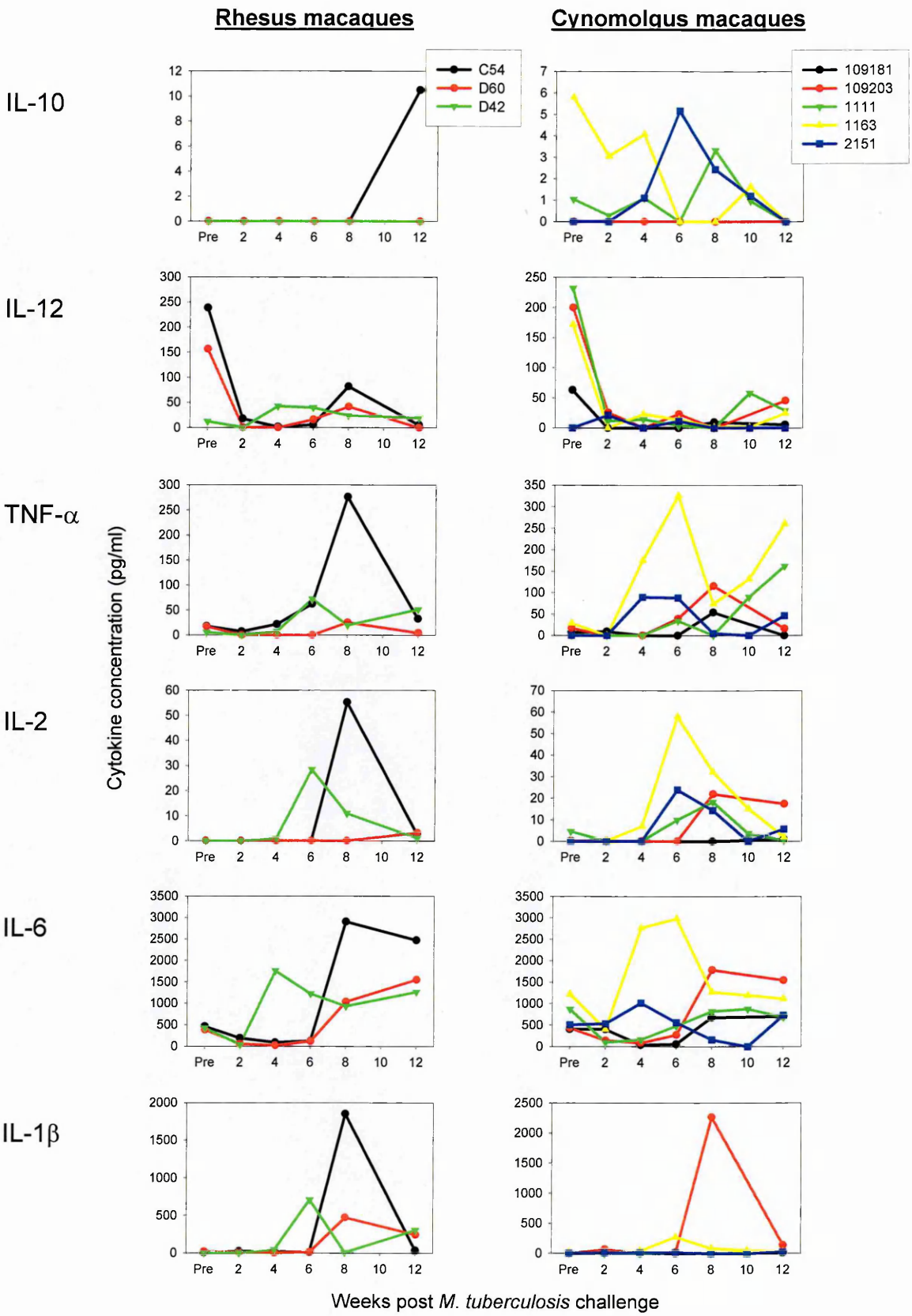


Figure 4.23: Secretion profiles of multiple cytokines in 3 day, PPD-stimulated PBMC supernatants after *M. tuberculosis* challenge.

4.3 DISCUSSION

4.3.1 Frequencies of IFN-γ-secreting cells post-challenge with *M. tuberculosis*

4.3.1.1 Responses in peripheral blood mononuclear cells

4.3.1.1.1 Antigen-specific responses

For all of the antigens tested (PPD and peptides to Ag85A, CFP10 and ESAT-6), responses were first detected 4 weeks post-challenge. This has previously been reported in other studies of *M. tuberculosis* infection using cynomolgus macaques (Lin *et al.*, 2006) and rhesus macaques (Gormus *et al.*, 2004). This is also in line with *M. bovis* infection in cattle (Dean *et al.*, 2005) and corresponds to specific protective immunity taking up to 3 weeks to become sufficient to control growth of *M. tuberculosis* (Toossi, 2000).

During peaks of response, the median frequencies of PPD-specific IFN-γ producing cells were approximately 3000 and 1000 IFN-γ-producing cells/10^6 PBMC in rhesus and cynomolgus macaques, respectively. In human studies levels ranged from 146-692 IFN-γ producing cells/10^6 PBMC (Abramo *et al.*, 2006; Leyten *et al.*, 2006; Nicol *et al.*, 2005). Thus, documented levels of PPD-specific IFN-γ-secreting cells in humans appear closer to the responses seen in the cynomolgus macaques than the rhesus macaques.

The median frequencies of IFN-γ-secreting cells to CFP10 and ESAT-6 were approximately 1500 and 2000 per 10^6 PBMC in rhesus macaques, respectively, and 300 per 10^6 PBMC for both antigens in the cynomolgus macaques. Human IFN-γ-secreting cell responses to CFP10 peptides ranged from 39-469/10^6 PBMC (Ewer *et al.*, 2006; Leyten *et al.*, 2006) and ESAT-6 peptides ranged from 18-390/10^6 PBMC (Ewer *et al.*,

2006; Leyten *et al.*, 2006; Pathan *et al.*, 2001). These levels are in line with the responses seen in the cynomolgus macaques as compared to the higher frequencies observed in the rhesus macaques.

However, although the data obtained from human studies appear to be more comparable to the levels detected in cynomolgus macaques rather than the rhesus macaques, caution in extrapolating similarities must be exercised. Firstly, responses in the non-human primates were measured for 12 weeks post-infection, whereas in the human studies infection would have lasted considerably longer. In most of the human studies patients were hospitalised or were confirmed as having TB by smear-positive culture, therefore these results do not reflect the early stages of disease progression. Secondly, the infectious dose of *M. tuberculosis* the animals received was known (approximately 40 cfu), and was given at a single time-point, whereas it has been estimated that the human infectious dose is 1-5 tubercle bacilli (Balasubramanian *et al.*, 1994b). In human infection, re-exposure is a distinct possibility or relapse of a previous infection may have occurred. Additionally, the strain of *M. tuberculosis* used in the macaques was known and would be different from that circulating in human populations. Different strains of *M. tuberculosis* have been shown to cause differences in immune response and virulence in mouse (Dunn & North, 1995) and rabbit (Manabe *et al.*, 2003) models of infection.

4.3.1.1.2 Comparison of IFN-γ-secreting cell frequencies in rhesus macaques and cynomolgus macaques post-challenge with M. tuberculosis

As the estimated dose of *M. tuberculosis* did not affect the levels of IFN-γ secreting cells detected, results from all of the infected rhesus macaques and cynomolgus macaques were directly compared after *M. tuberculosis* infection. The data showed that rhesus

macaques have significantly higher frequencies of antigen-specific IFN-γ-secreting cells than cynomolgus macaques during early infection with *M. tuberculosis*.

After *M. tuberculosis* challenge, a range of frequencies of IFN-γ-secreting cells was detected in the animals. This heterogeneity of data has been reported in cynomolgus macaques before, due to the genetic diversity of non-human primates giving degrees of animal-to-animal variation (Lin *et al.*, 2006). Animal-to-animal variation has also been seen in the cattle model through the course of *M. bovis* infection (Dean *et al.*, 2005). Therefore, due to the outbred nature of the macaques it is not surprising that there is variation seen in the levels of IFN-γ-secreting cells.

4.3.1.1.3 Effect of IFN-γ-secreting cell frequencies on rapid disease progression

When responses in four rhesus macaques that were euthanised due to meeting humane endpoints (caused by rapid disease progression) were compared to the animals which survived to 12 weeks post-infection, no significant differences in frequency of IFN-γ-secreting cells were found for any of the antigens tested.

Three of the four animals that met humane endpoints received higher doses of *M. tuberculosis* (D53, 500 cfu; D12, 75 cfu; D19, 70 cfu), whereas the other rhesus macaques received 30-40 cfu. Therefore, a higher dose had an impact on the severity of disease, but not on IFN-γ production. When different doses of *M. bovis* have been used to infect calves, varying from 1,000 to 1 cfu, there were no difference in the time taken to achieve a positive IFN-γ response or levels of IFN-γ responses (Dean *et al.*, 2005). This observation is similar with the IFN-γ responses seen in *M. tuberculosis*-challenged macaques.

4.3.1.1.4 Responses to individual peptides post-M. tuberculosis challenge

Responses from individual peptide pools were analysed to determine whether any pools contained consistently recognised epitopes or whether the target of the immune response changed through the course of early infection.

Results from the CFP10 peptide pools show that pools 2 and 3 are the most frequently detected in macaque infection. In human infection $CFP10_{71-85}$ has been identified as having multiple epitopes (Shams *et al.*, 2004). The equivalent peptide is number 13 in our peptide set. Thus, although the whole peptide is in pool 3, due to the overlapping nature of the peptide sequences some of the sequence overlaps into pool 2. Thus, it is possible that this same region is also immunogenic in macaque infection.

For ESAT-6, pool 1 was the most dominant in rhesus macaques and pool 3 in cynomolgus macaques. Results from human studies show strikingly similar results to those of the macaques, with the beginning and end of the ESAT-6 sequence being the most immunogenic (Pathan *et al.*, 2001; Ulrichs *et al.*, 2000).

Responses to Ag85A peptide pools are more difficult to interpret as pools are not made up of sequential peptides. However, an interesting observation was that peptide pool D was responsive in all but one of the 18 macaques. It has been reported that the amino acid region from 61-80 gives the strongest proliferative response in TB patients (Launois *et al.*, 1994). This 20mer peptide is represented by peptides 12-15 using 15mers, which are found in pools A, D, E and G. Although pool D is highly immunogenic, it would be speculative to suggest that this is due to this specific peptide region and further work would have to be done to clarify responses in more detail.

4.3.1.1.5 *Decline in IFN-γ-secreting cells at 6-8 weeks post-infection*

An unexpected observation was the decline in responses in most animals 6-8 weeks post-challenge with *M. tuberculosis*. As this phenomenon was seen in animals from different challenge experiments, it is unlikely that this could be an experimental artefact.

It has been reported by other researchers that antigen-specific T lymphocytes are sequestered at the site of disease, thus making responses appear anergic in the peripheral circulation. Evidence for this includes IFN-γ levels in pleural fluid being 65-fold higher than in plasma in TB patients (Hirsch *et al.*, 2001). Also, more PPD-specific IFN-γ-secreting cells have been detected in the lung compartment of patients with TB as compared with responses in the blood (Breen *et al.*, 2006).

Alternatively, an increase in Th2 cytokines may account for a decrease in Th1 cytokine, including IFN-γ, activity. In one study, decreased IFN-γ production was associated with increased IL-4 production (Smith *et al.*, 2002). However, the long stimulation period (7 days) may have allowed the immune profile to shift from the *in vivo* situation. Another report showed that in mice challenged with *M. tuberculosis* by tail vein, a peak of IFN-γ-secreting cells was followed by a peak of IL-4-producing cells (Orme *et al.*, 1993b). Therefore, there is increasing evidence that both Th1 and Th2 arms of the immune system may be involved in the response to *M. tuberculosis,* and this may be particularly evident in those patients with more extensive disease (Wilsher *et al.*, 1999). However, not all researchers agree with the Th2 contribution to mycobacterial immunity. Using mRNA expression of PBMC from TB patients, expression of Th1 cytokines (IFN-γ and IL-2) was diminished, but no change in mRNA expression was detected for the Th2 cytokines (IL-4, IL-10, and IL-13), thus showing no association between Th1 and Th2

responses at the level of systemic T cells (Zhang *et al.*, 1995). Therefore, the impact of Th2 cytokines on Th1 cytokine responses remains controversial in TB immunity.

Apoptosis of IFN-γ-secreting cells may also cause reductions in frequencies of IFN-γ-secreting cells (Hirsch *et al.*, 1999). Continuous exposure to *M. tuberculosis*-infected antigen-presenting cells may cause programmed cell death of *M. tuberculosis*-specific effector cells resulting in the inability to restimulate central memory cells and to prime naïve precursors (Goletti *et al.*, 2006), especially during chronic infection (Lazarevic *et al.*, 2005a). Apoptosis in macrophages may benefit the immune response by releasing apoptotic vesicles that carry mycobacterial antigens to MHC class I and CD1-restricted T cells (Schaible *et al.*, 2003). Additionally, apoptosis reduces mycobacterial growth, as seen by a 60-70% loss in viability of intracellular BCG of apoptotic cells *in vitro* (Molloy *et al.*, 1994). Ironically, IFN-γ may be involved in the mediation of apoptosis as although it is immunoprotective in the sense that it increases the ability of macrophages to contain mycobacteria, it also contributes to apoptosis in responsive T cells (Hirsch *et al.*, 2001). Thus, IFN-γ is essential to a regulatory mechanism that eliminates activated $CD4^+$ T cells and maintains $CD4^+$ T cell homeostasis during an immune response (Dalton *et al.*, 2000). In the macaques studied, there was no detectable loss in lymphocytes (including $CD3^+$, $CD4^+$, and $CD8^+$), granulocytes or monocytes (appendices 3-8, sections 8.3-8.8). The observation of no changes in the number of $CD4^+$ and $CD8^+$ T-lymphocytes has also been observed in the rhesus macaques and cynomolgus macaques post-*M. tuberculosis* challenge by others (Langermans *et al.*, 2001), therey proving little evidence of apoptosis in the macaque model.

Anergy of effector cells may also cause fewer cells to produce IFN-γ after antigenic stimulation. Anergy generally follows activation of individual T cells (Yamamoto *et al.*,

2007), so after the peak of IFN-γ response cells may become unresponsive. However, proliferation results show that the PBMC are still capable of expanding *in vitro* when IFN-γ responses are decreased (appendices 9-10, sections 8.9-8.10), thus arguing against anergy causing a depression in immune function. This proliferation in the absence of IFN-γ production has also been reported by others (Launois *et al.*, 1994; Onwubalili *et al.*, 1985; Surcel *et al.*, 1994), providing evidence that the cells are capable of multiplying after antigenic stimulation, and so are unlikely to be anergic.

Activated regulatory T cells are also known to suppress IFN-γ production (Becker *et al.*, 2007; Roberts *et al.*, 2007). Regulatory T cells have been seen at sites of infection in the spleen and lung in mice, and been shown to prevent eradication of tubercle bacilli by suppressing an otherwise efficient $CD4^+$ T cell response (Kursar *et al.*, 2007). Their role during *M. tuberculosis* infection is not clear. It is possible that they are programmed by specific microbial stimuli, thus ensuring survival of tubercle bacilli (Kursar *et al.*, 2007). Conversely, suppression of the immune response could serve as a means of maintaining a constant antigenic pool providing a continuous supply of antigen-specific T cells to defend against reinfection, or as a means of curtailing active pathology (Kursar *et al.*, 2007).

Alternatively, the reduction in response may be due to cycling bacterial antigen levels (Murray, 1999) causing bursts and reductions in the immune response (Lazarevic *et al.*, 2005a). Additionally, antigen-presenting cells, such as dendritic cells, may have less ability to present antigens during ongoing disease (Aleman *et al.*, 2007) affecting the immune response. Due to the study period only being 12-weeks post-challenge, further cycling of responses may become evident during the progression of TB disease.

4.3.1.1.6 Responding T cell subsets

The majority of IFN-γ was secreted by $CD4^+$ cells, as depletion of this subset reduced responses and enrichment (by depletion of $CD8^+$ cells) increased proportions of IFN-γ-secreting cells. It is expected that $CD4^+$ T cells are the principal cells involved in the immune response to mycobacteria, as bacterial antigens can find their way into the class II MHC processing pathway and are subsequently presented to $CD4^+$ T cells (Murray, 1999). For PPD-specific responses, only $CD4^+$ cells were stimulated to produce IFN-γ. This has also been reported by others who demonstrated that PPD was able to stimulate $CD4^+$ lymphocytes, but not $CD8^+$ T lymphocytes (Barry *et al.*, 2003).

In a single rhesus macaque and a single cynomolgus macaque, there was evidence of peptide-specific $CD8^+$ IFN-γ secretion. The contribution of $CD8^+$ T cells to IFN-γ production is affected by several factors. When they are killing infected cells, as measured by the secretion of perforin, their ability to produce cytokines is compromised (Sad *et al.*, 1996). However, when $CD8^+$ T cells are stimulated with higher numbers of APC their ability to produce cytokines is greatly increased, probably because of repeated stimulation and/or stimulation of a greater proportion of $CD8^+$ T cells (Sad *et al.*, 1996). These observations have been confirmed by levels of T-cell receptor (TCR) occupancy affecting cytotoxic T lymphocyte (CTL) responses. IFN-γ production required a substantial level of TCR occupancy, whereas cytotoxicity was elicited by triggering fewer TCRs (Valitutti *et al.*, 1996). Although these observations were made *in vitro*, these features probably occur *in vivo* whereby they provide a regulatory mechanism. When infection levels are high the $CD8^+$ T cells contribute to the cytokine response to attempt to bring *M. tuberculosis* infection under control, with $CD8^+$ T cells contributing equally to IFN-γ production (Lazarevic *et al.*, 2005a). However, once the infection levels

have been reduced their cytokine production is not necessary, but their cytolytic function is still needed to clear residually infected cells.

4.3.1.2 Responses in local lymphoid tissues

As lung material was not available (due to requirements for MRI scanning analysis and histology), the hilar lymph nodes represented the second site of disease. In this study, responses in the hilar lymph nodes in macaques were generally lower than those seen in PBMC. This is in contrast to studies in cynomolgus macaques where, during early infection, responses were broader and stronger in the hilar lymph nodes than in PBMC (Lin *et al.*, 2006). The difference may be due to those investigators using a different route of challenge, as they achieved infection by instilling *M. tuberculosis* via a bronchoscope. Alternatively, the cynomolgus macaques in the report by Lin *et al.* were euthanised 3-6 weeks post-infection, whereas in this study the cynomolgus macaques were assessed later in infection at 12 weeks post-*M. tuberculosis* challenge.

Responses in the axillary lymph nodes were measured in order to determine levels at a site more distant from infection. Although, as expected, responses were much lower it was still apparent that some antigen-specific responses were being detected. This may be due to the axillary lymph node trapping some antigen-presenting cells that passed by or escaped from the hilar lymph node.

The spleen is able to trap antigen-specific lymphocytes that escape the lymphatic system and enter the systemic circulation (Chackerian *et al.*, 2002). This may in turn account for the spleen also being a site of secondary *M. tuberculosis* infection as it traps infected cells which then multiply and spread the infection to this organ. In both rhesus and cynomolgus macaques, antigen-specific IFN-γ-secreting cells were detected in the

spleen. This is in contrast to a study in 4 rhesus monkeys where dissemination of *M. tuberculosis* to the spleen was not detected 12 weeks post-challenge (Lewinsohn *et al.*, 2006). However, these animals were challenged by instillation of bacteria via bronchoscope using a higher dose (1000 cfu) and a different strain (H37Rv) of *M. tuberculosis*.

Analysis was performed to determine whether frequencies of IFN-γ-secreting cells in PBMC and lymphoid tissue correlated with disease severity. Spleen responses in rhesus macaques increased alongside the severity of infection as determined by the extent of lung involvement measured using the lesion:lung volume ratio. In the rhesus macaques, there was a strong correlation between responses in the spleen when using Ag85A and ESAT-6 peptides. No correlation was observed in the cynomolgus macaques. However, in the cynomolgus macaques only one animal had a high lesion:lung volume ratio and increased IFN-γ frequencies, so the data were less evenly distributed. Looking at bacteria load in the spleen results showed detectable *M. tuberculosis* infection in 7 spleens from 9 rhesus macaques, but in the cynomolgus macaques infection was observed in only 2 spleens from 9 animals (appendix 11, section 8.11). Haematogenous spread has been reported for *M. tuberculosis* infection in rhesus macaques by others after 10 (Anacker *et al.*, 1972) and 13 (Barclay *et al.*, 1970) weeks post-infection. Where the two species have previously been compared, there was miliary spread to the spleen in the rhesus macaques but not in the cynomolgus macaques 9 weeks post-infection (Langermans *et al.*, 2001). Therefore, in the cynomolgus macaques the infection does not spread into the spleen.

Surprisingly, in neither species did responses in the hilar lymph nodes correlate with disease severity. As this is the draining lymph node from the primary site of infection in the lungs, it would be expected to show a strong correlation. When bacteriology was performed on the hilar lymph nodes, there was a high bacterial load in these tissues

(appendix 12, section 8.12). Thus, it may be that the infection in the lymph nodes had overwhelmed the capacity of this tissue to generate a sufficient protective immune response. This has been seen in guinea pigs aerosolly infected with *M. tuberculosis* where the lymph node architecture had been destroyed (Kraft *et al.*, 2004).

4.3.2 Concentrations of IFN-γ secreted from circulating cells post-infection with *M. tuberculosis*

4.3.2.1 Whole blood stimulation

IFN-γ levels in whole blood stimulated for 6 days were detected in supernatants 6 weeks after *M. tuberculosis* challenge. In whole blood stimulated for 5 days, IFN-γ was detected in household contacts when they sero-converted to disease (Whalen *et al.*, 2006). This is similar to our assay, where disease was clearly evident by analysis of chest X-rays at 4 weeks post-infection (appendix 13, section 8.13). When responses in 6-day whole blood supernatants were compared to the overnight ELISPOT results, little correlation was seen between the two readouts. The main differences in the assays were the time of stimulation (overnight for ELISPOT, 6 days for whole blood stimulation) and the cell preparation used (PBMC for ELISPOT, whole blood for stimulation assay). Additionally, the ELISA only measures secreted cytokine present in the supernatant, whereas the ELISPOT assay captures it as soon as it is produced preventing its uptake by other cells. Another factor is that by using whole blood, the number of cells used are not pre-determined. However, immunophenotyping results (appendices 3-8, sections 8.3-8.8) showed no major alterations in numbers of circulating immune cells. Other investigators have also found that IFN-γ production does not correlate with parameters such as whole blood counts or granulocyte numbers (Sahiratmadja *et al.*, 2007b).

To determine whether the difference in responses was due to stimulation times, diluted whole blood was incubated overnight in agreement with the ELISPOT assay. The results showed a positive correlation, which has also been shown by others who stimulated whole blood overnight (Goletti *et al.*, 2005). This indicates that with the 6 day supernatants a different response was being detected due to the longer period of stimulation. This may be due to increased $CD8^+$ contributions due to the prolonged incubation allowing optimal antigen uptake and presentation (Serbina & Flynn, 1999). Alternatively, compared with effector memory cells, central memory cells need a longer period of stimulation to produce effector cytokines (Sallusto *et al.*, 1999). The contribution of central memory cells may thus relate to differences in IFN-γ secretion.

4.3.2.2 PBMC stimulation

PBMC's were stimulated for 3 days with PPD, as used by macaque studies (Langermans *et al.*, 2001; Langermans *et al.*, 2005). Responses started four weeks post-challenge, in line with reports on cynomolgus macaques (Langermans *et al.*, 2005). There was not a close correlation between the amount of IFN-γ secreted and the frequencies of IFN-γ-producing cells. Mean macaque responses were between 5000-7500 pg/ml. In human patients, PBMC responses after 3-4 days of stimulation with heat-killed *M. tuberculosis* were 327 (far advanced disease)-1,639 pg/ml (moderately advanced disease) (Sodhi *et al.*, 1997). However, results cannot be directly compared with macaque data due to differences in antigen preparation and the history of clinical disease is not fully known.

4.3.2.3 Comparison of rhesus macaques and cynomolgus macaques

When responses from the different stimulation studies were compared, there were no consistent differences in the amount of IFN-γ secreted between the two macaque species.

This is an interesting observation, as previously the frequency of IFN-γ cells after *M. tuberculosis* infection have been shown to be higher in rhesus macaques as compared to cynomolgus macaques. Therefore, it could be postulated that cynomolgus macaques may have less cells secreting IFN-γ, but each cell secretes cytokine more efficiently, thus maintaining the same concentrations of IFN-γ released.

4.3.2.4 Measurement of other cytokines

To determine whether other cytokines had an effect on IFN-γ secretion, luminex analysis was carried out to simultaneously measure multiple cytokines in a single sample. To ensure that this assay gave similar results to the standard ELISA assay, a comparison of the two assays was made using IFN-γ. A strong correlation between the two assays was found, although the actual concentrations reported were 20-fold lower for the luminex. Therefore, although trends were comparable, concentrations were not.

From the luminex results, TNF-α production peaked at weeks 6-8, coinciding with the peaks in secretion of IFN-γ. TNF-α affects the expression of many chemokines, and therefore plays a significant role in granuloma formation (Algood *et al.*, 2004) and is a critical cytokine that is essential for early control of primary infection (Botha & Ryffel, 2003). IL-2 production also peaked at weeks 6-8 alongside IFN-γ secretion in both rhesus macaques and cynomolgus macaques. This observation has also been noted in $CD3^+$ T cells in patients with active pulmonary TB (Winkler *et al.*, 2005), in serum cytokine levels (Berktas *et al.*, 2004; Dlugovitzky *et al.*, 1997), and in bronchoalveolar fluid (Casarini *et al.*, 1999). IL-6 was produced in all three rhesus macaques studied post-challenge with *M. tuberculosis* and in 2 of the 5 cynomolgus macaques. However, production of IL-6 was not related to levels of IFN-γ secreted. Other investigators have found that IL-6 production is enhanced with IFN-γ secretion (Orme *et al.*, 1993b; Verbon

et al., 1999). However, IL-6 can also differentiate activated regulatory T cells into IL-17-producing cells (Xu *et al.*, 2007). Therefore, due to its range of functions in early infection its effects may not be influenced by IFN-γ. IL-1β was produced by all three rhesus macaques and two of the five cynomolgus macaques tested. Mice lacking the receptor for this cytokine have been shown to be more susceptible to pulmonary TB infection and for a decreased ability of splenocytes to produce IFN-γ (Juffermans *et al.*, 2000). The peaks of IL-1β levels coincided with IFN-γ secretion in the macaques, therefore suggesting that secretion levels between these two cytokines are related.

In addition to those cytokines being measured via luminex, IL-10 and IL-12 concentrations were measured by ELISA assay, due to non-human primate reagents not being available at the time for analysis in the luminex assay. IL-10 concentrations were measured due to the ability of this cytokine to reduce IFN-γ secretion (Gong *et al.*, 1996). IL-10 was only detected in one out of three rhesus macaques and above background in two out of five cynomolgus macaques at 6 and 8 weeks post-infection. This is the same point at which the frequencies of IFN-γ-secreting cells decline in most animals. Therefore, although this observation was only observed in a minority of animals, it could be speculated that IL-10 may potentially play a role in down-regulation of IFN-γ-producing cells. IL-12 levels in the supernatants were difficult to assess as the sample at pre-bleed was high in all of the animals. Therefore, post-challenge results rarely exceeded those detected at baseline. However, this observation could be due to the sequestration of IL-12 secreting cells to the site of disease in the lungs. Other investigators have reported that IL-12 secretion is compartmentalised in the lung (Morosini *et al.*, 2003), thus detection in PBMC proves difficult.

4.4 CONCLUSION

This is the first time that rhesus macaques and cynomolgus macaques responses have been simultaneously monitored after aerosol *M. tuberculosis* infection. After challenge, the IFN-γ responses showed differences between the two macaque species, with rhesus macaques having more cells secreting IFN-γ after mycobacteria-antigen stimulation, yet both species secreting the same concentration of IFN-γ. Analysis of the responses during the early time-points after challenge have shown that levels IFN-γ did not provide a marker for rapid disease progression.

5 IFN-γ RESPONSES AFTER IMMUNISATION WITH BCG AND BCG/MVA85A

5.1 INTRODUCTION

Having analysed IFN-γ responses during early disease progression in *M. tuberculosis* infection, the next aim was to analyse responses after vaccination. In addition to measuring the dynamics of IFN-γ production after vaccinating macaques, responses after challenge of vaccinated animals with *M. tuberculosis* were also assessed to determine whether IFN-γ measurements could be used as correlates of protection following TB vaccination. A correlate of protection may not necessarily be part of the mechanism of protection, so looking at protective vaccination responses may be critical. For example, a new vaccine may induce protective immunity without engaging the pathways contributing to protection against naturally acquired *M. tuberculosis* (Ellner *et al.*, 2000). The ideal scenerio is to find a surrogate marker of protection for *M. tuberculosis*, where many populations and different settings are generalised to be protected (Sadoff & Wittes, 2007).

5.1.1 Vaccination of macaques against TB

Rhesus macaques were used to test the current human vaccine, BCG, as early as in 1955 (Schmidt, 1972). Up until the mid-1970's several researchers working on simian TB reported the protective effects of BCG vaccination on *M. tuberculosis* infection in rhesus macaques. Thereafter, reports in 2001 and 2002 (Langermans *et al.*, 2001; Shen *et al.*, 2002) have assessed BCG-induced protection in rhesus macaques. All of these reports

are summarised in table 5.1. In summary, these reports show that in 8 of the 9 studies BCG conferred some protection against TB disease in rhesus macaques.

As well as different routes of vaccination, different strains of BCG were also used in the non-human primate studies by the different researchers (Good, 1968; Ribi *et al.*, 1971). It was generally observed that BCG did not completely protect the rhesus macaques by giving sterilising immunity, but instead resulted in a lesser extent of pathological disease. Due to many of these studies being carried out before modern immunological techniques had been widely introduced, the primarily readouts were pathology scores, clinical signs and occasionally basic immunology assessments (antibody production or proliferation assays).

Author ref	**Route of BCG administration**	**Protected against *M. tuberculosis*?**
(Good, 1968)	Intravenous	Yes (apart from Kikuth's isoniazid-resistant strain)
(Barclay *et al.*, 1970)	Intravenous	Yes
(Ribi *et al.*, 1971)	Intravenous	Yes
(Anacker *et al.*, 1972)	Intramuscular or intravenous	Yes
(Schmidt, 1972)	Intratracheal, intracutaneous or intravenous	Yes
(Barclay *et al.*, 1973)	Aerosol or intravenous	Yes
(Janicki *et al.*, 1973)	Intravenous and intracutaneous	Yes
(Langermans *et al.*, 2001)	Intradermally	No
(Shen *et al.*, 2002)	Intravenous	Yes

Table 5.1: Summary of papers on BCG vaccination conferring protection against *M. tuberculosis* challenge in rhesus macaques.

Following the 1970s there has been very little published work on tuberculosis research in non-human primates. More recently, however, work has begun again in macaque studies of infection and vaccination against *M. tuberculosis*. BCG vaccination has been assessed in rhesus macaques for expansion of lymphocyte subsets (Lai *et al.*, 2003), though animals were not challenged with virulent *M. tuberculosis*. The contribution of γδ T cells has also been assessed in a rhesus macaque model during BCG vaccination and after *M. tuberculosis*-infection (Shen *et al.*, 2002), showing the potential importance of this cell phenotype for the clearance of mycobacteria.

Following the early work in rhesus macaques, recent experiments have begun to use the cynomolgus macaque model. Vaccines tested in cynomolgus macaques include heat-shock protein (HSP) 65 combined with human IL-12 DNA within a haemagglutinating virus of Japan (HVJ)-liposome (Kita *et al.*, 2005; Okada *et al.*, 2007) and a recombinant BCG expressing the 72f fusion gene (Kita *et al.*, 2005) – all giving survival equal or better than BCG alone.

Only one study has compared immune responses in rhesus macaques and cynomolgus macaques directly using both BCG vaccine and *M. tuberculosis* infection (Langermans *et al.*, 2001). However, this study used a large dose of 3,000 cfu delivered via intratracheal instillation to achieve disease in both species, which is unrepresentative of natural human infection. Unlike earlier studies performed decades before, the rhesus macaques failed to achieve any substantial protection in this study, although this may have been due to the treatment of the macaques with anti-mycobacterial antibiotics to clear any residual BCG bacteria (Langermans *et al.*, 2001). The only IFN-γ readout presented was concentration of IFN-γ secreted at a single time-point post-vaccination with BCG.

5.1.2 Modified vaccinia Ankara virus expressing Ag85A as a booster vaccine

In addition to BCG vaccination, the BCG and MVA85A prime-boost regime was tested in macaques to determine responses to a novel TB vaccine that is undergoing human clinical trials. MVA85A has entered phase II clinical trials in South Africa (Sander & McShane, 2007), where it has been shown to be safe and immunogenic (Hawkridge *et al.*, 2008). This vaccine has also shown great promise in trials conducted in West Africa (Brookes *et al.*, 2008) and the UK (McShane *et al.*, 2004). Due to there being no pre-defined immunological correlates of protection against *M. tuberculosis*, determining whether the significantly enhanced immune responses observed after MVA85A boost are accompanied by an improvement in protective efficacy is a key question (Pathan *et al.*, 2007). To evaluate this in humans would be expensive and time consuming, as vaccinees would need to be followed for many years to determine whether there was a correlation between their IFN-γ response and their subsequent susceptibility to TB infection. Therefore, by using the macaque model, a controlled infection could be delivered post-vaccination to determine the efficacy of a vaccine in an animal model that very closely resembles human infection.

5.1.3 Chapter aims

The main aims of this chapter are:

1) To determine IFN-γ responses after vaccination with BCG in non-human primates.
2) To assess IFN-γ responses after BCG vaccination followed by responses after MVA85A, a novel TB vaccine currently undergoing human clinical trials.
3) To monitor responses after vaccinated and non-vaccinated macaques are challenged with *M. tuberculosis* and determine whether IFN-γ measurements are indicative of survival.
4) To compare data on responses obtained in macaques with data generated in human clinical trials.

5.1.4 Chapter methods

In a pilot study, 2 rhesus macaques and 2 cynomolgus macaques were vaccinated with BCG and responses monitored for 74 weeks (section 2.1.3.2). A larger study of rhesus macaques were analysed after BCG vaccination and BCG/MVA85A immunisation, after which animals were challenged via aerosol with *M. tuberculosis* to determine protective efficacies (section 2.1.3.3).

The frequency of antigen-specific IFN-γ secreting cells was measured using the ELISPOT method (section 2.2.6) and the concentration of IFN-γ secreted after antigenic stimulation was measured using the ELISA method (section 2.2.7). Anti-vector antibodies to MVA85A vaccination were measured in sera samples using an anti-vaccinia ELISA assay (section 2.2.9).

5.2 RESULTS

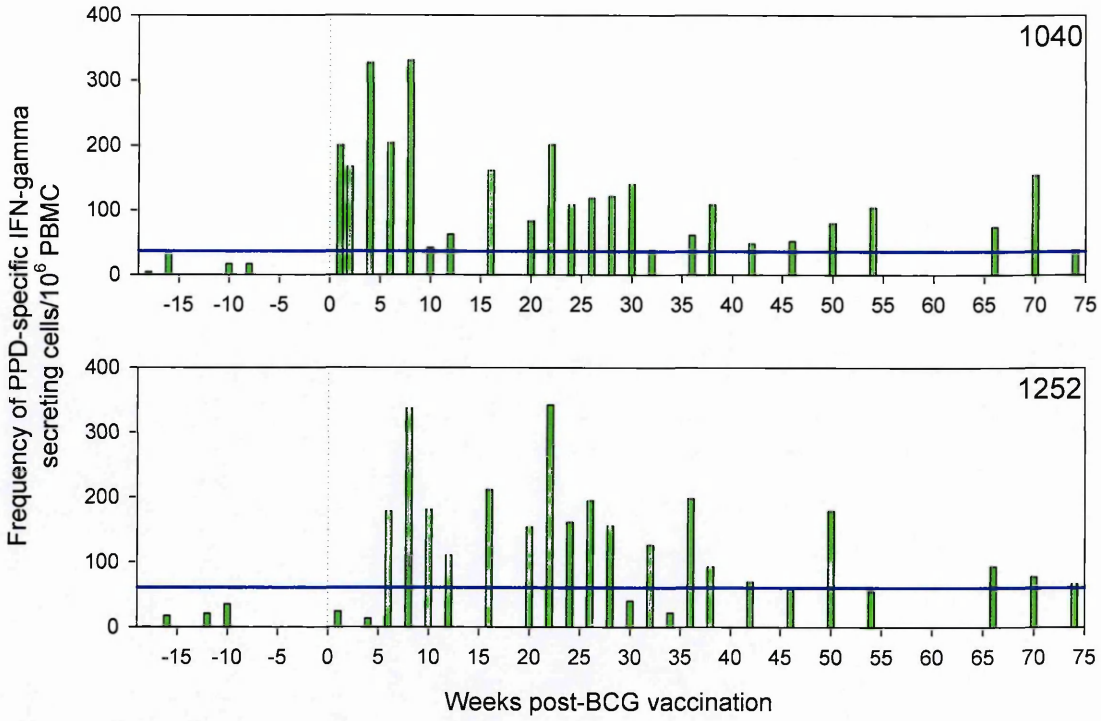
5.2.1 IFN-γ responses post-vaccination with BCG

5.2.1.1 Comparison between rhesus macaques and cynomolgus macaques

5.2.1.1.1 Frequency of IFN-γ secreting cells

In a preliminary experiment, two rhesus and two cynomolgus macaques were vaccinated with BCG vaccine. The frequencies of PPD-specific IFN-γ-secreting cells were measured over the 74 week post-BCG vaccination period (figure 5.1). Rhesus macaques show PPD-specific responses peaking at around 350 IFN-γ-secreting cells at week 4 in animal 1040 and week 8 in animal 1252. Responses were detected throughout the 74 weeks post-vaccination. In contrast, the BCG-vaccinated cynomolgus macaques displayed lower frequencies of PPD-specific IFN-γ-secreting cells. In animal 9911021, all responses were below the cut-off value. In animal 1007 no responses were detected at pre-bleed, so using a cut-off of 3 times the pre-bleed value was zero. When mean responses were plotted (figure 5.2), the frequencies of PPD-specific IFN-γ-secreting cells were consistently lower in cynomolgus macaques than in rhesus macaques.

Rhesus macaques



Cynomolgus macaques

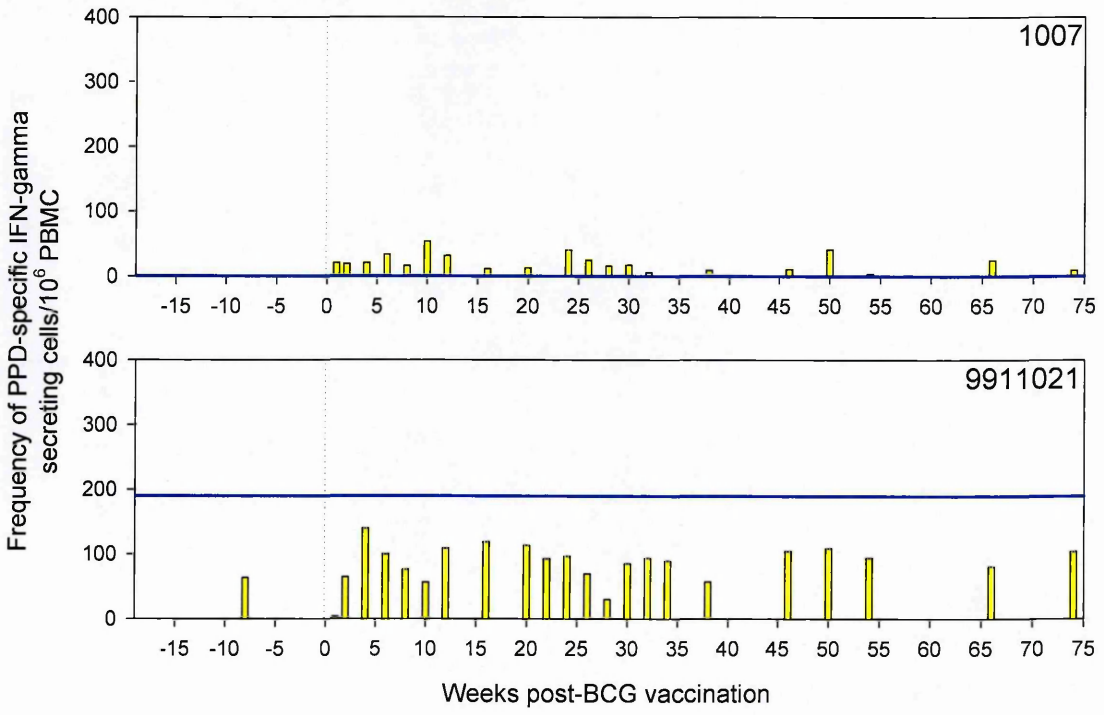


Figure 5.1: Frequencies of PPD-specific IFN-γ-secreting cells post-BCG vaccination in macaques.

(Rhesus macaques, green bars; cynomolgus macaques, yellow bars. Animal identification number is shown in top right corner of each graph. Blue line indicates cut-off of 3 times the mean pre-bleed level).

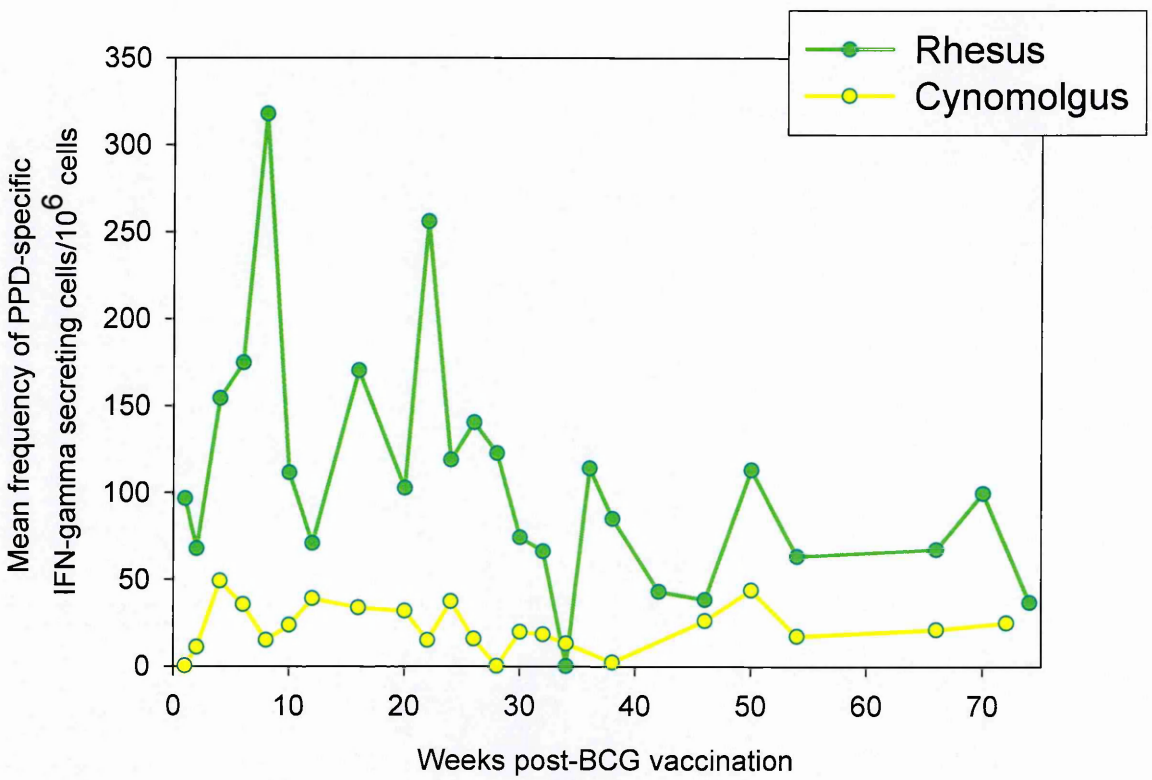


Figure 5.2: Mean frequencies of PPD-specific IFN-γ-secreting cells in macaques.

(Rhesus macaques, green line; cynomolgus macaques, yellow line).

With a distinct difference between the frequencies of PPD-specific IFN-γ-secreting cells in the two macaque species, data from human clinical studies conducted in Oxford were used to determine which, if either, more closely resembled the responses detected in humans.

As shown in figure 5.3, the responses from rhesus macaques more closely follow those seen in humans than do the levels observed in the cynomolgus macaques. The frequencies of PPD-specific IFN-γ-secreting cells in both the humans and the rhesus macaques peaked at 8 weeks post-vaccination with BCG. In contrast, the lower frequencies of antigen-specific IFN-γ-secreting cells detected in the cynomolgus macaques did not resemble the responses seen in human studies.

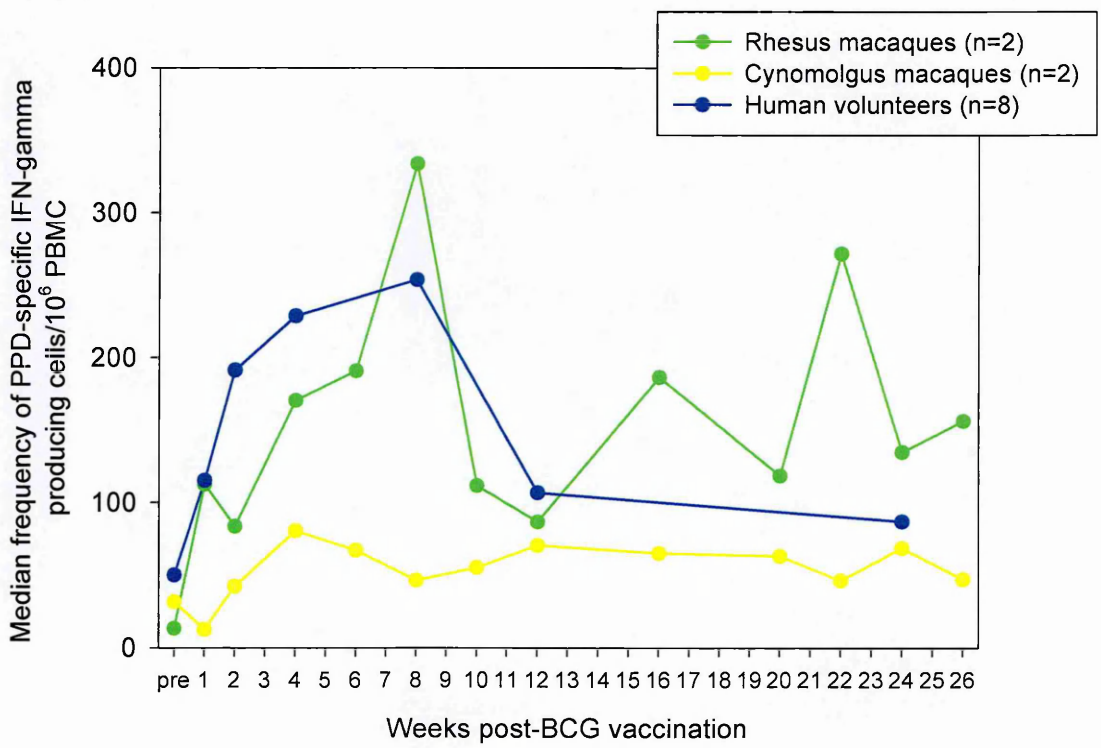


Figure 5.3: Median frequencies of PPD-specific IFN-γ-secreting cells post-vaccination with BCG in macaques and human volunteers.

(Rhesus macaques, green line; cynomolgus macaques, yellow line; and human volunteers, blue line).

5.2.1.1.2 Concentration of IFN-γ secreted

In addition to measuring the frequencies of IFN-γ-secreting cells after BCG vaccination in the rhesus macaques and cynomolgus macaques, the concentration of PPD- and BCG-specific IFN-γ release after stimulation of diluted whole blood for 6 days was also measured.

As with the frequencies of IFN-γ-secreting cells, the concentration of IFN-γ released was lower in cynomolgus macaques than in rhesus macaques after BCG vaccination (figure 5.4). Peak responses were detected 6 weeks post-BCG vaccination in the two rhesus macaques, and at weeks 6 and 10 in the cynomolgus macaques. However, in the cynomolgus macaque, levels in animal 1007 remained mainly below the cut-off value set for the assay. When samples were initially measured, the peak response in animal 1252 was over the limit of detection of the assay (3000 pg/ml). Therefore, the supernatant was diluted 1:5 and re-analysed. The diluted sample gave a value which was still over the limit of detection (3874 pg/ml). Therefore, the actual concentration is estimated from this value as no further supernatant was available for re-testing.

Responses to PPD antigen were generally higher than with BCG in the rhesus macaques, whereas the reverse was true for the cynomolgus macaques. When the responses to the PPD and BCG antigens were compared using the Spearman's rank correlation statistical test there was a significant correlation between antigens in both rhesus macaques (r_s=0.746, P<0.001) and cynomolgus macaques (r_s=0.654, P=0.001). As PPD is used as an antigen in the ELISPOT assay, for future work only responses against this antigen were subsequently tested.

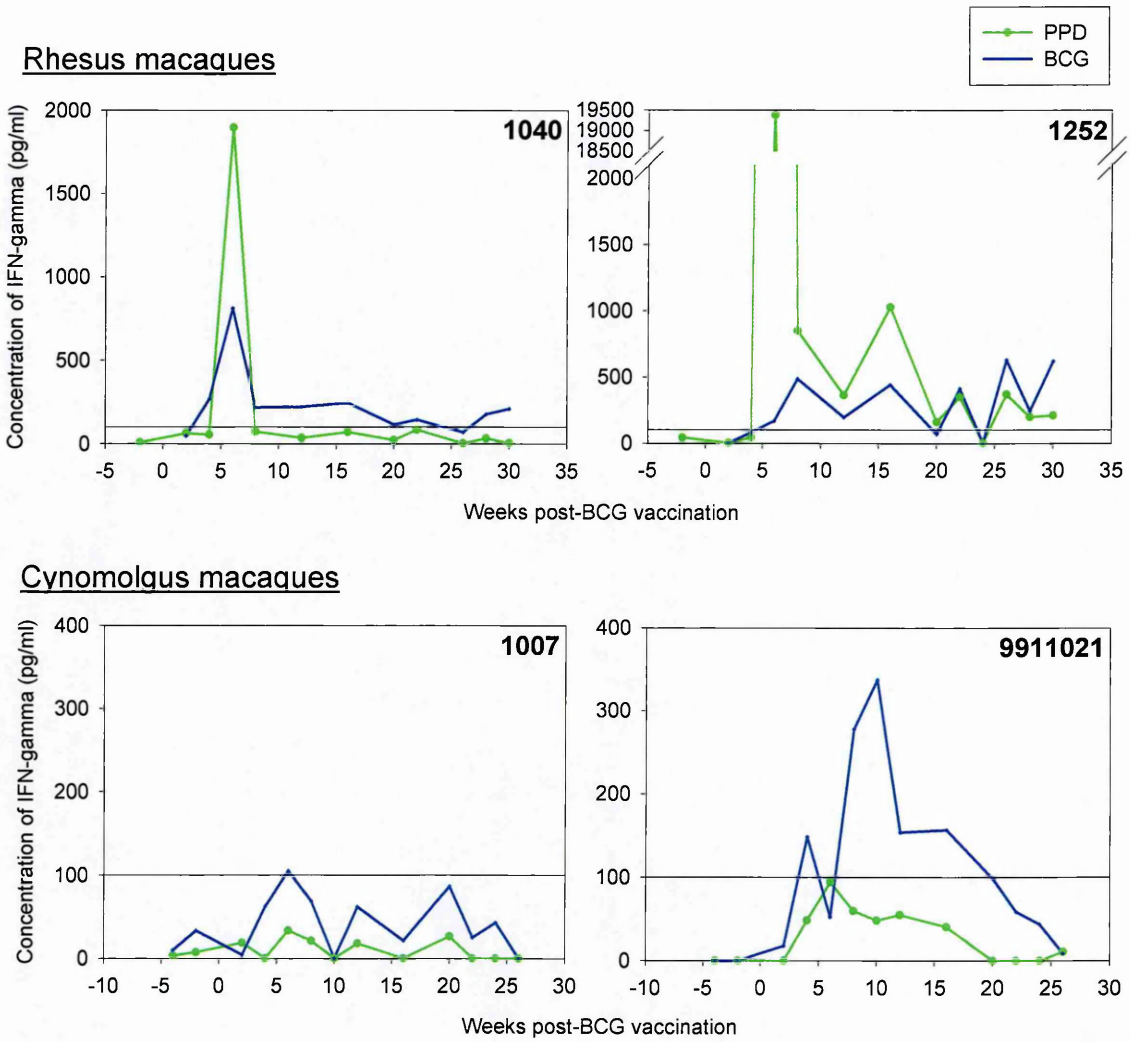


Figure 5.4: Concentration of IFN-γ secreted in PPD-stimulated and BCG-stimulated 6-day diluted whole blood supernatants in rhesus macaques and cynomolgus macaques. (PPD stimulated, green line; BCG stimulated, blue line. Animal identification numbers are displayed in the top right corner of each graph. Reference line indicates cut-off level; 100 pg/ml).

5.2.1.2 Frequencies of IFN-γ-secreting cells in BCG-vaccinated rhesus macaques

The observation of responses in rhesus macaques being similar to human responses was followed up in an increased sample size of rhesus macaques. Twelve rhesus macaques were vaccinated intradermally in the upper arm with BCG vaccine and the frequencies of IFN-γ-secreting cells in response to stimulation with PPD and Ag85A peptide pools were measured. In parallel, responses in four unvaccinated rhesus macaques were measured simultaneously as a control. Samples were measured every 2 weeks after BCG vaccination up to 12 weeks (section 2.1.3.3).

As shown in figure 5.5, significant differences in levels of PPD-specific IFN-γ secreting cells between BCG-vaccinated and unvaccinated rhesus macaques were seen from 4 weeks post-vaccination with PPD antigen. However, few Ag85A-specific IFN-γ-secreting cells were detected in BCG-vaccinated animals. Responses in the unvaccinated control animals remained low to both the PPD and the Ag85A peptide pool antigens.

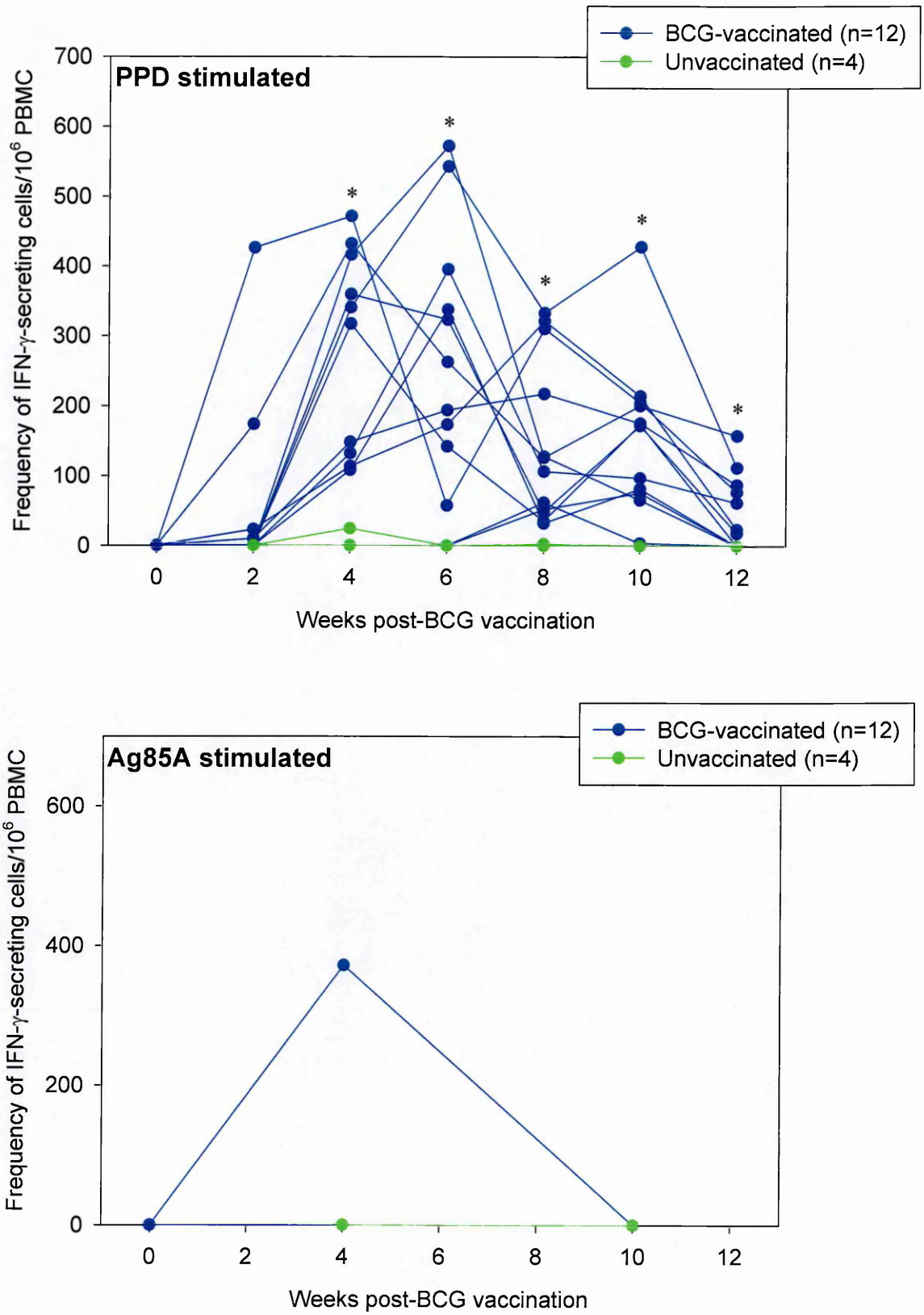


Figure 5.5: Frequencies of PPD-specific and Ag85A-specific IFN-γ-secreting cells above cut-off in rhesus macaques vaccinated with BCG and unvaccinated controls (BCG vaccinated, blue lines; unvaccinated, green lines. Cut-off defined as 3 times the mean pre-bleed value. * = significant difference [Mann-Whitney statistical value $P<0.05$]).

To determine whether the profiles of PPD-specific IFN-γ-secreting cells in the twelve BCG-vaccinated rhesus macaques were similar to human responses, data were compared with those from a human clinical trial conducted in Oxford where eight volunteers were vaccinated with the same volume and strain of BCG with responses similar in both studies. When median results were plotted (figure 5.6) the rhesus macaques and human studies showed comparable frequencies of PPD-specific reactivity. However, it is not known whether the peak frequencies of IFN-γ-secreting cells in the rhesus macaque group at 6 weeks post-vaccination is mirrored by humans as levels were not measured at this time-point in the latter. No significant differences between rhesus macaques and human responses were observed post-BCG vaccination (Table 5.2).

Mann-Whitney statistical p-value	Weeks post-BCG vaccination			
	2	**4**	**8**	**12**
Rhesus macaques vs. Humans	0.3749	0.2170	0.8471	0.8471

Table 5.2: Mann-Whitney statistical analysis of frequencies of IFN-γ-secreting cells in rhesus macaques and humans after vaccination with BCG.

(Significance, $P<0.05$).

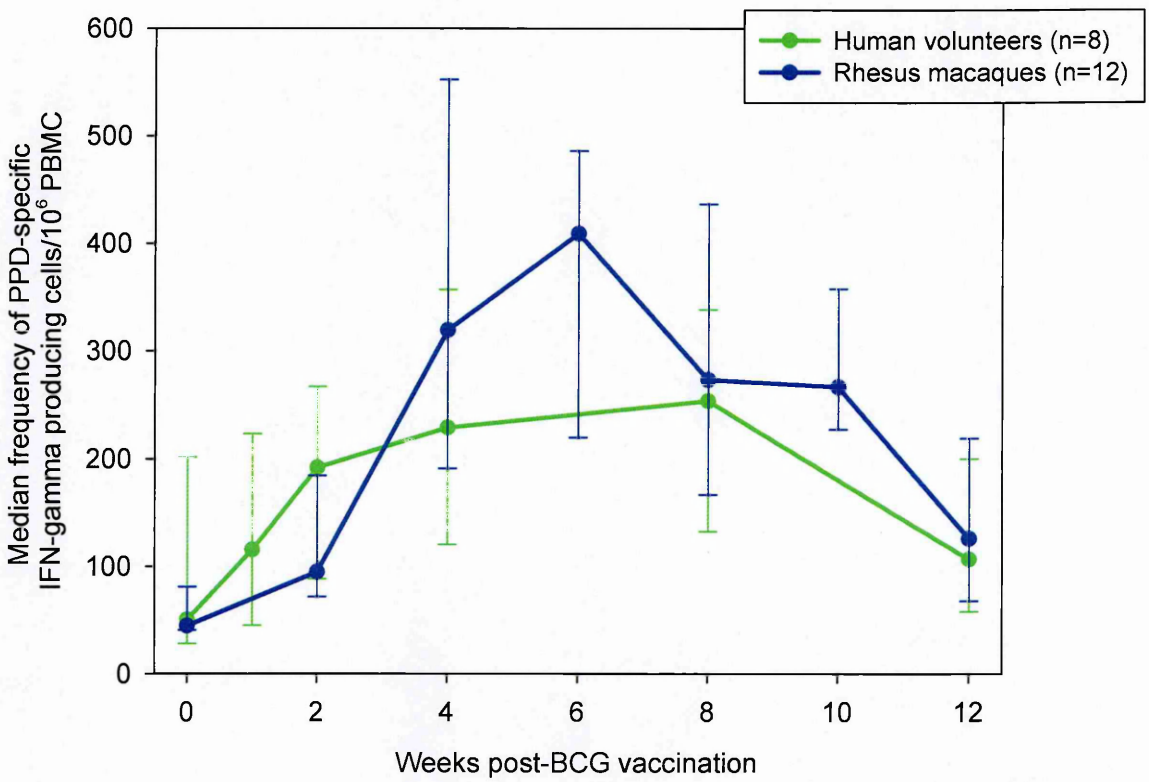


Figure 5.6: Median values of IFN-γ-secreting cells in BCG-vaccinated rhesus macaques and human volunteers.

(Rhesus macaques, blue line; human volunteers, green line. Error bars indicate interquartile range of samples).

5.2.1.3 Concentrations of IFN-γ secreted in diluted whole blood supernatants from BCG-vaccinated rhesus macaques

The concentration of IFN-γ secreted in diluted whole blood after 6-day stimulation with PPD was measured in the BCG-vaccinated rhesus macaques.

As shown in figure 5.7, PPD-specific IFN-γ production was seen after vaccination with BCG in 7 of the 12 animals above cut-off levels (animals K59, K50, K44, K61, K65, K79 and K86). None of the unvaccinated controls showed any PPD-specific IFN-γ secretion above cut-off levels during the 12 week time-course. Using area under the curve analysis, a significant difference (P=0.0064) was found between BCG-vaccinated and unvaccinated rhesus macaques (figure 5.8).

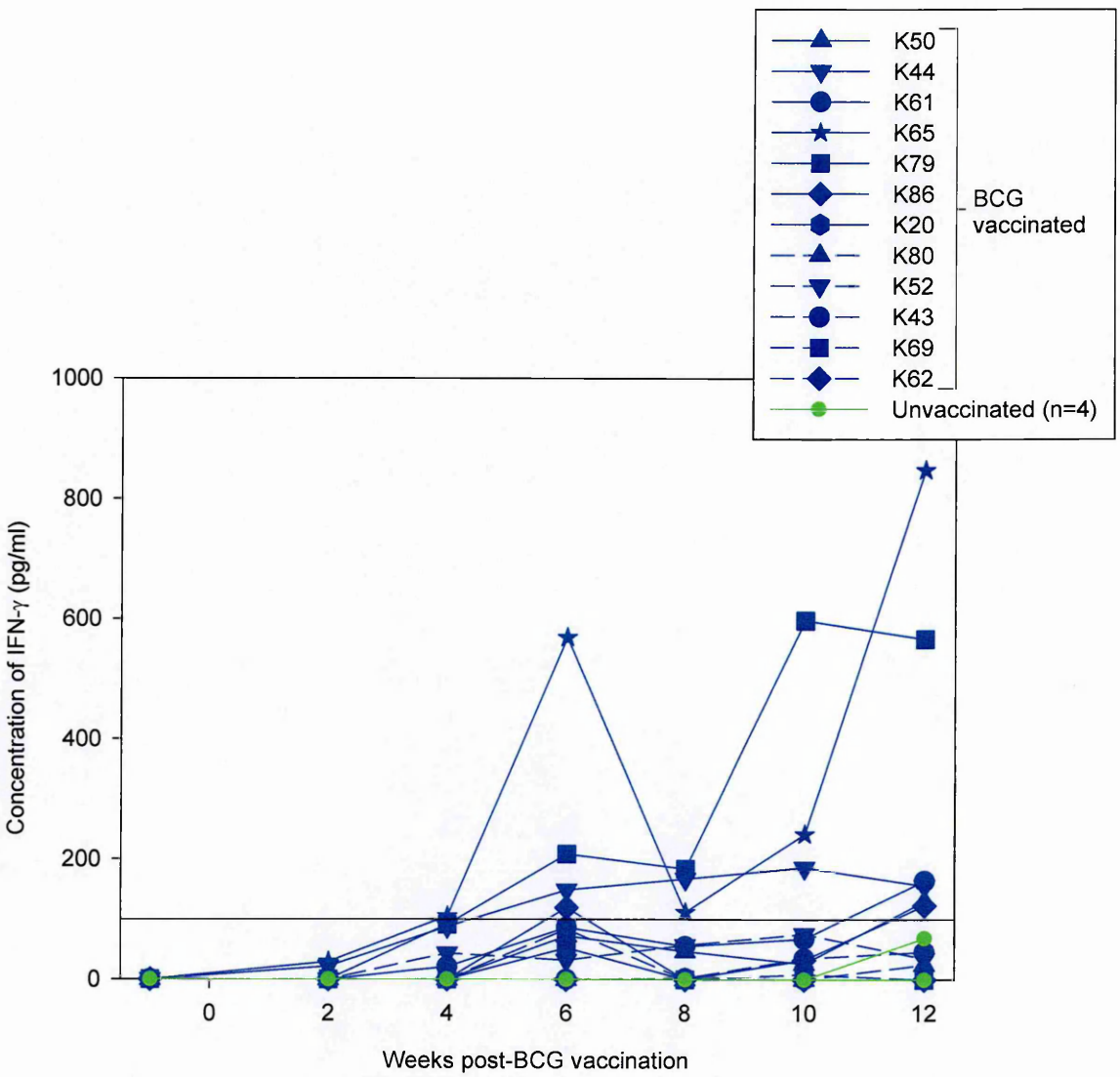


Figure 5.7: Concentrations of IFN-γ secreted in diluted whole blood supernatants from rhesus macaques vaccinated with BCG compared to unvaccinated controls.

(BCG vaccinated, blue lines; unvaccinated, green lines. Reference line indicates cut-off; 100 pg/ml).

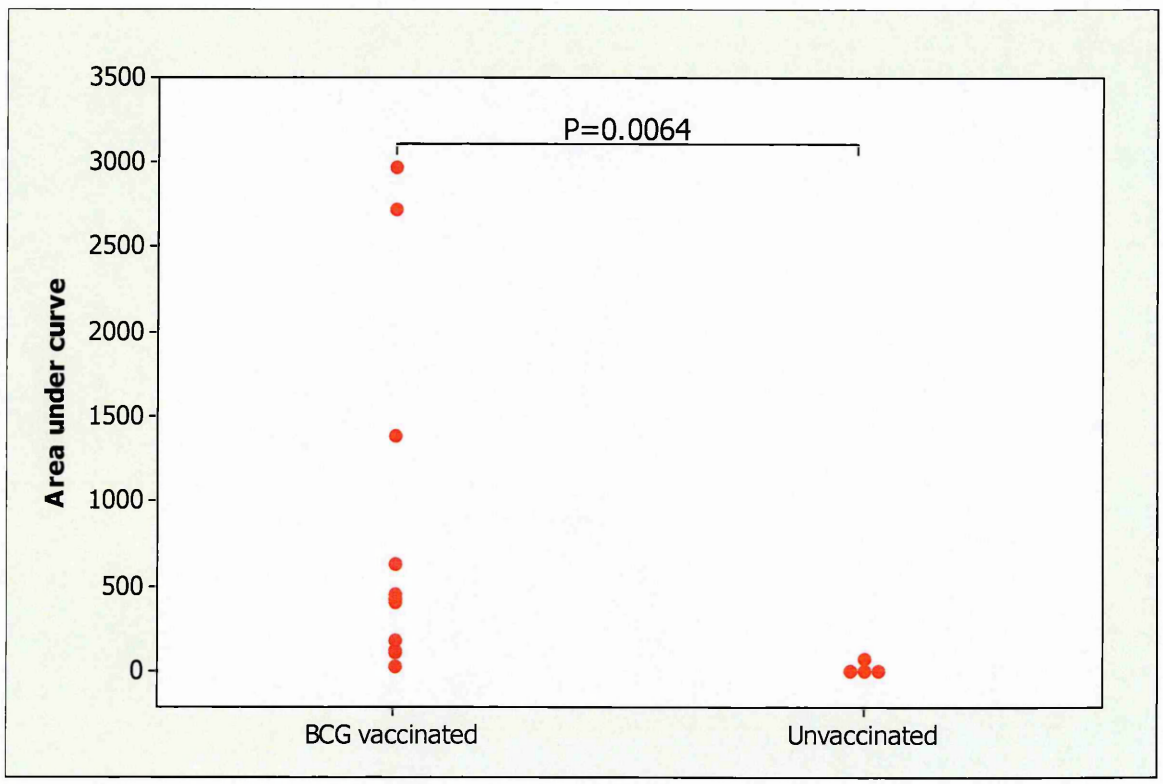


Figure 5.8: Area under the curve analysis showing differences in amount of IFN-γ secreted between BCG-vaccinated and unvaccinated rhesus macaques.

(Analysis carried out using a Mann-Whitney statistical test. Significance, $P<0.05$).

As the concentration of IFN-γ secreted in response to stimulation with PPD was measured alongside the frequencies of PPD-specific IFN-γ-secreting cells, a statistical analysis was carried out to determine if the two measurements were significantly correlated.

Using the Spearman's rank correlation test, there was no significant correlation between the two assays (r_s=0.425). As seen from figure 5.9, high levels of IFN-γ were secreted in the ELISA assay when only moderate frequencies of IFN-γ-secreting cells were detected in the ELISPOT assay. In addition, there were also times where the ELISA assay failed to detect any IFN-γ secretion yet the ELISPOT assay showed that frequencies of IFN-γ-secreting cells were detected.

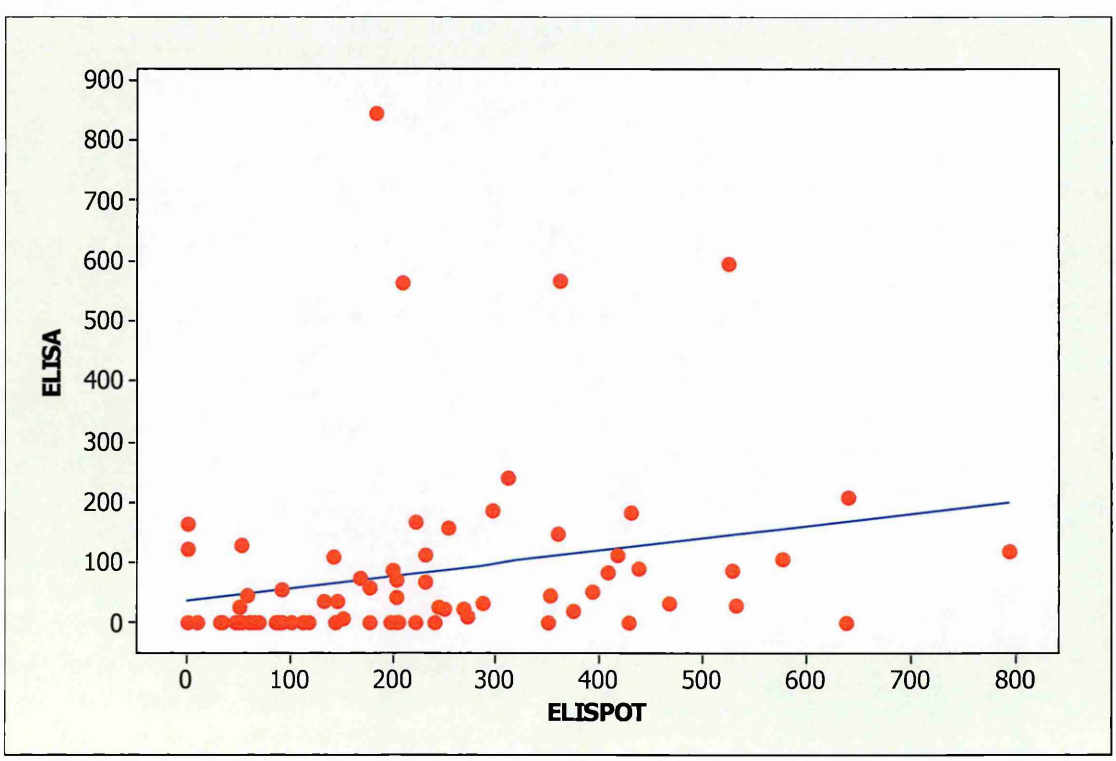


Figure 5.9: Comparison of PPD-specific responses in concentrations of IFN-γ secreted after stimulation of diluted whole blood with the frequencies of IFN-γ-secreting cells detected in PBMC after vaccination of rhesus macaques with BCG.

5.2.2 Responses after immunisation of BCG-vaccinated animals with MVA85A

Of the twelve animals vaccinated with BCG, six animals were immunised using the MVA85A vaccine. IFN-γ responses were monitored in these animals over an 8 week period after immunisation with MVA85A.

5.2.2.1 Frequencies of IFN-γ secreting cells post-MVA85A immunisation

Post-MVA85A immunisation, the frequency of PPD- and Ag85A-specific IFN-γ-secreting cells increased in 5/6 and 6/6 of the animals, respectively (figure 5.11). One animal (K20) showed no boosting of IFN-γ-secreting cells with PPD.

Responses peaked one week after MVA85A immunisation in most of the animals. Animal K79 proved an exception as Ag85A-specific responses peaked 2 weeks post-MVA85A immunisation and PPD-specific responses increased until week 6. Animal K65 showed much higher frequencies of PPD- and Ag85A-specific IFN-γ secreting cells following MVA85A immunisation in comparison to the other five MVA85A-immunised animals.

Six weeks after MVA85A immunisation there were no significant differences in frequency of antigen-specific IFN-γ-secreting cells detected between the MVA85A-immunised animals and those that had not been MVA85A-immunised.

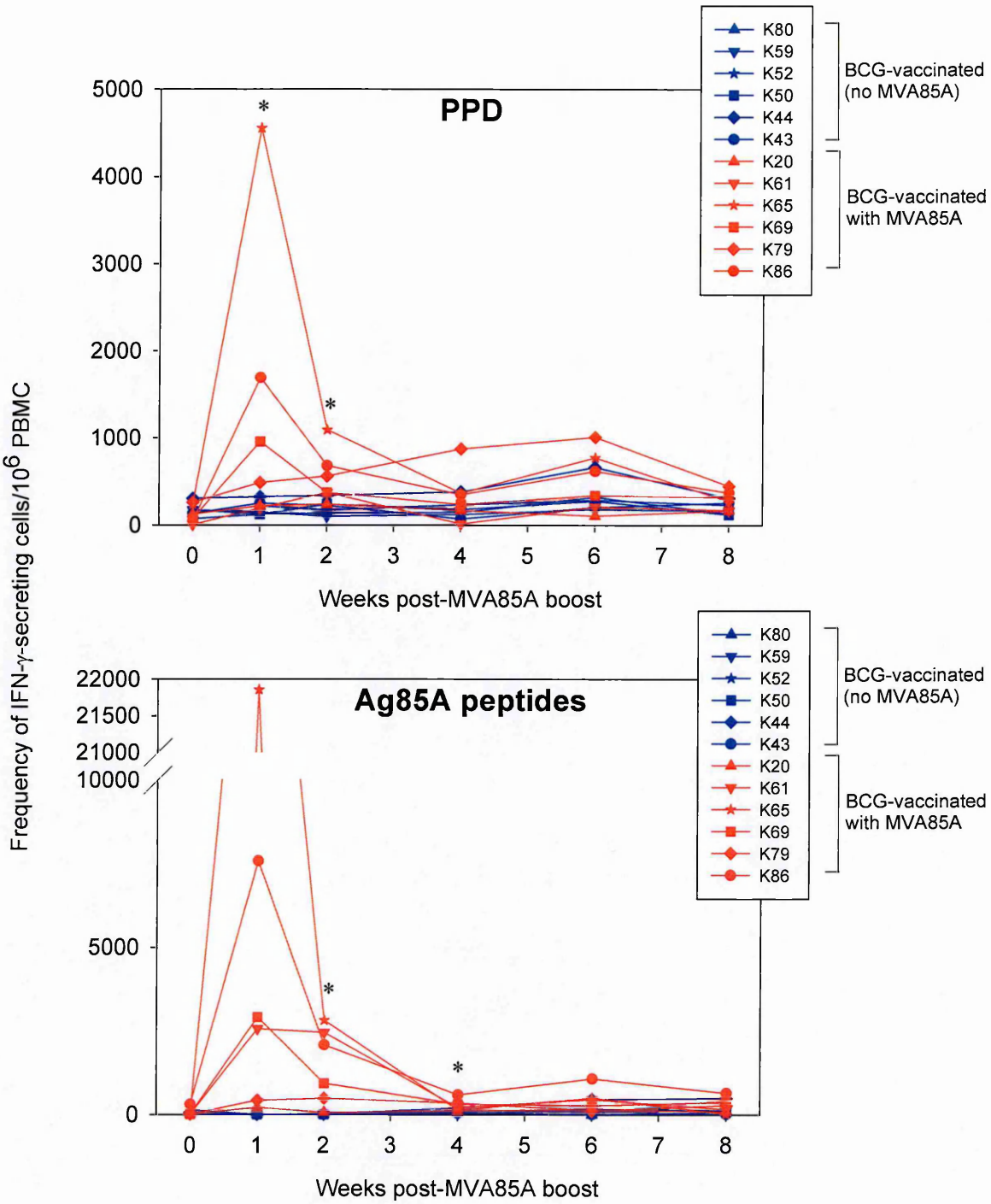


Figure 5.10: Frequencies of PPD-specific and Ag85A-specific IFN-γ-secreting cells post-MVA85A immunisation in rhesus macaques previously vaccinated with BCG compared with unboosted controls.

(MVA85A-immunised, red lines; BCG-vaccinated only, blue lines. Numbers beginning with 'K' refer to animal identification. * = significant difference [Mann-Whitney statistical test, $P<0.05$]).

Responses from the MVA85A-immunised rhesus macaques were compared with data where MVA85A had been used in clinical trials to boost BCG-vaccination responses in human volunteers (Oxford trial T009, Helen McShane, personal communication). However, the rhesus macaques received $5x10^8$ pfu whereas the human volunteers received $1x10^8$ pfu MVA85A (Beveridge *et al.*, 2008). When the median responses between the rhesus macaques were compared with data from the human studies (figure 5.11), the kinetics of response were extremely similar. Both groups showed peaks at 1 week post-MVA85A boost with a decline thereafter.

Statistical analysis of the results showed no significant differences between peak responses 1 week post-MVA85A immunisation in rhesus macaques as compared with humans (table 5.3). With PPD, there were no statistically significant differences in response during the 8 weeks post-MVA85A immunisation between rhesus macaques and humans. However, with the Ag85A peptides there were significant differences after the initial response at week 1, with human volunteers maintaining a higher number of Ag85A-specific IFN-γ-secreting cells despite receiving a lower dose of MVA85A. When the area under the curve was measured post-MVA85A vaccination in humans and rhesus, there was no statistical difference with either antigen tested over the 8 week study period (figure 5.12).

Mann-Whitney statistical p-value	Rhesus macaques vs. humans	
	PPD	Summed Ag85A peptides
Week 1	0.3254	0.2061
Week 2	0.2061	0.0131
Week 4	0.2061	0.0009
Week 8	0.4824	0.0009

Table 5.3: Comparison of frequencies of IFN-γ-secreting cells post-MVA85A immunisation in rhesus macaques and human clinical trials.

(Mann-Whitney statistical test; significance level, $P<0.05$).

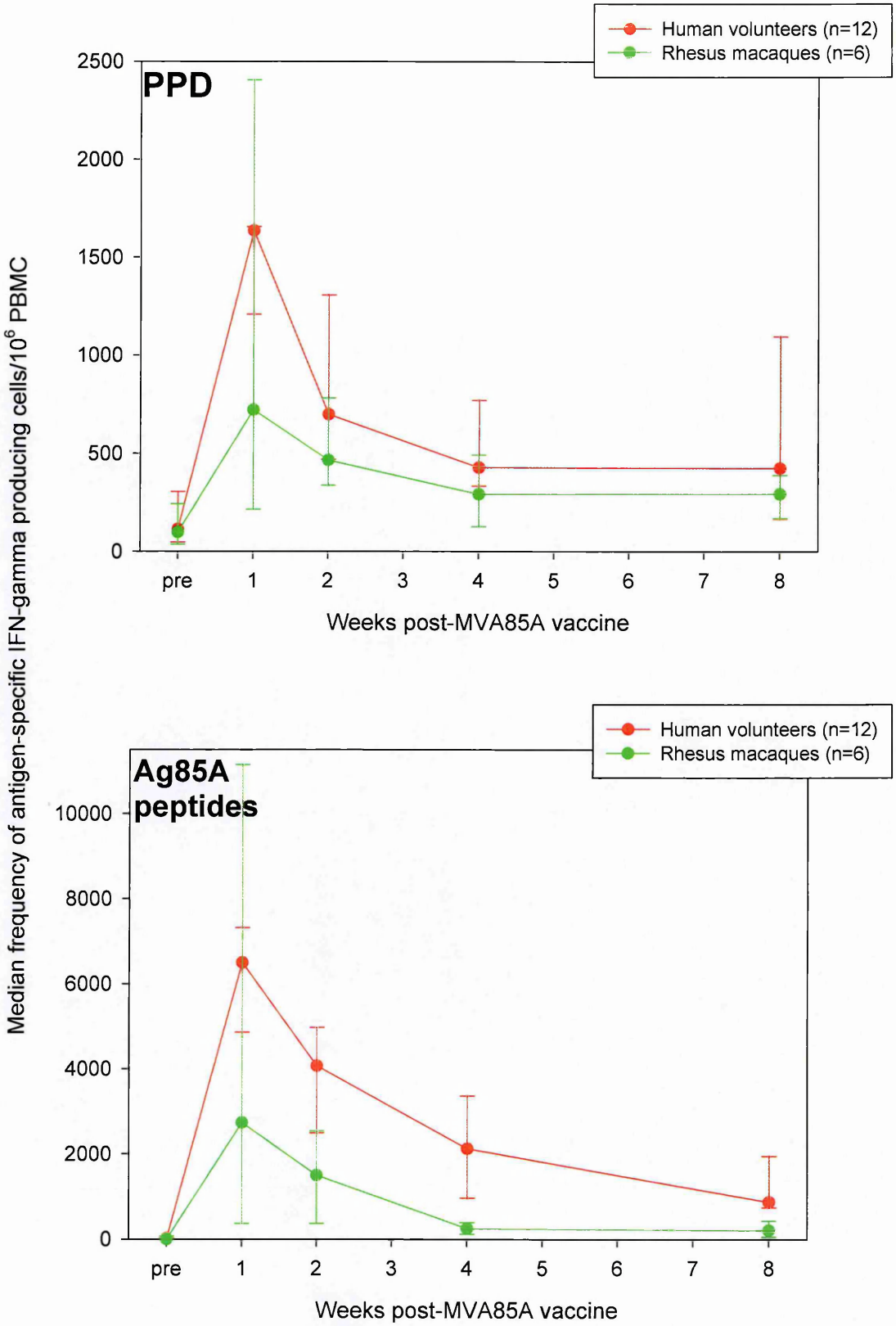


Figure 5.11: Comparison of mean frequencies of PPD-specific and Ag85A-specific IFN-γ-secreting cells post-MVA85A immunisation of rhesus macaques and human volunteers.

(Rhesus macaques, green line; human volunteers, red line. Error bars denote interquartile range of samples).

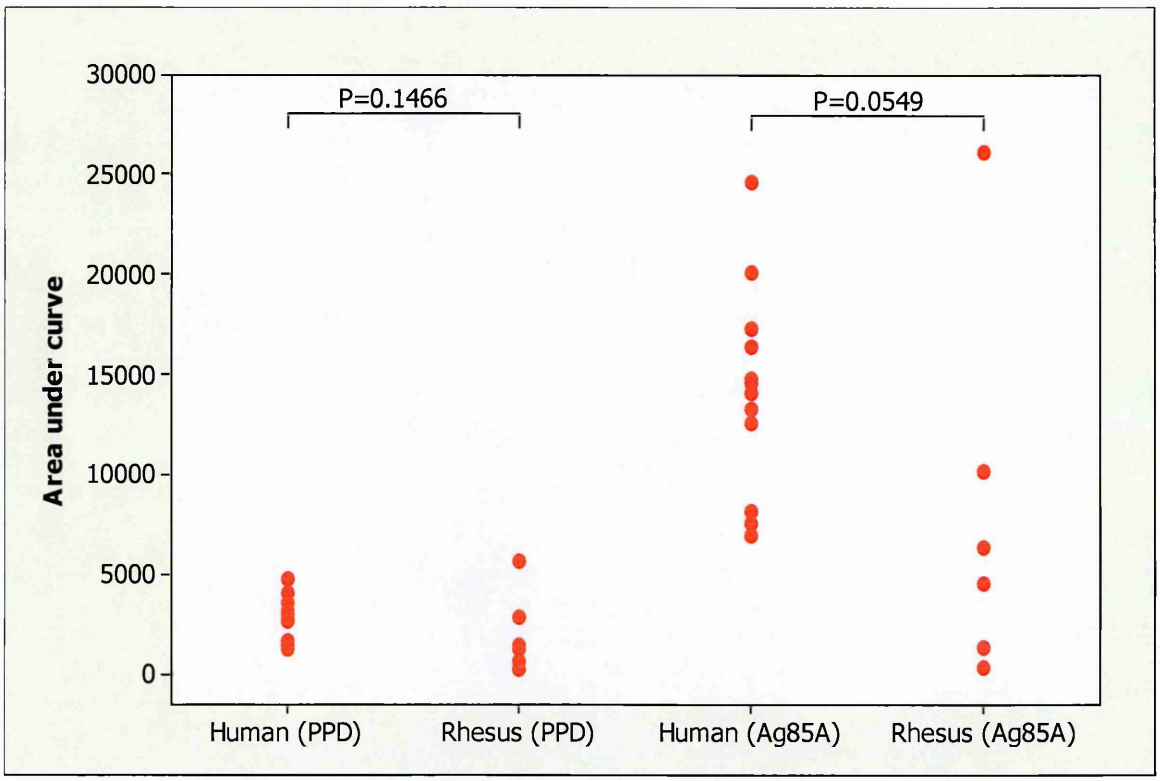


Figure 5.12: Area under curve analysis after MVA85A immunisation comparing responses in humans and rhesus macaques using PPD and Ag85A peptides. (Mann-Whitney statistical test, significance level $P<0.05$).

5.2.2.2 Concentration of IFN-γ secreted after immunisation of BCG-vaccinated rhesus macaques with MVA85A

The concentration of IFN-γ secreted in PPD-stimulated 6-day diluted whole blood supernatants was measured in BCG-vaccinated animals immunised with MVA85A and in BCG-only controls. As shown in figure 5.13, half of the animals that received MVA85A showed a peak response 1 week post-immunisation (K69, K86, K65), indicating that the boost was successful. The remaining three MVA85A-immunised animals (K20, K79, K61) failed to show any increase in PPD-specific IFN-γ production and were therefore not boosted.

Graphical analysis showed a trend in differences between the BCG/MVA85A-immunised and BCG-only animals post-MVA85A boost. However, using the Mann-Whitney statistical test at each timepoint (table 5.4) or by analysing areas under the curve (figure 5.14), the differences were non-significant ($P>0.05$) confirming that the MVA85A had not resulted in a boosted immune response.

Mann-Whitney statistical p-value	Weeks post-MVA85A boost					
	0	**1**	**2**	**4**	**6**	**8**
MVA85A immunised vs. BCG-only	0.4320	0.0538	0.0741	0.0741	0.2840	0.0633

Table 5.4: Comparison of concentrations of IFN-γ secreted in animals immunised with MVA85A compared to BCG-only controls.

(Mann-Whitney statistical test; significance level, $P<0.05$).

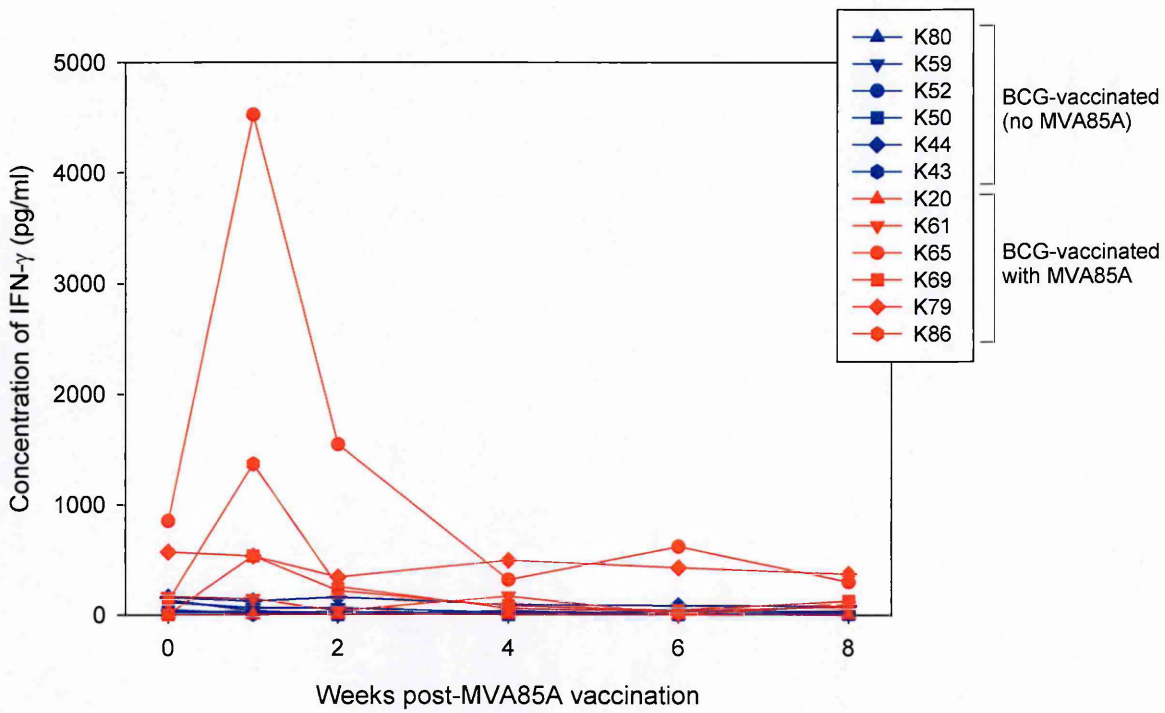


Figure 5.13: Concentration of PPD-specific IFN-γ secretion in rhesus macaques immunised with MVA85A compared with BCG-only controls.

(BCG/MVA85A-immunised, red lines; BCG-only, blue lines. Numbers beginning with 'K' corresponds to individual animal identification numbers).

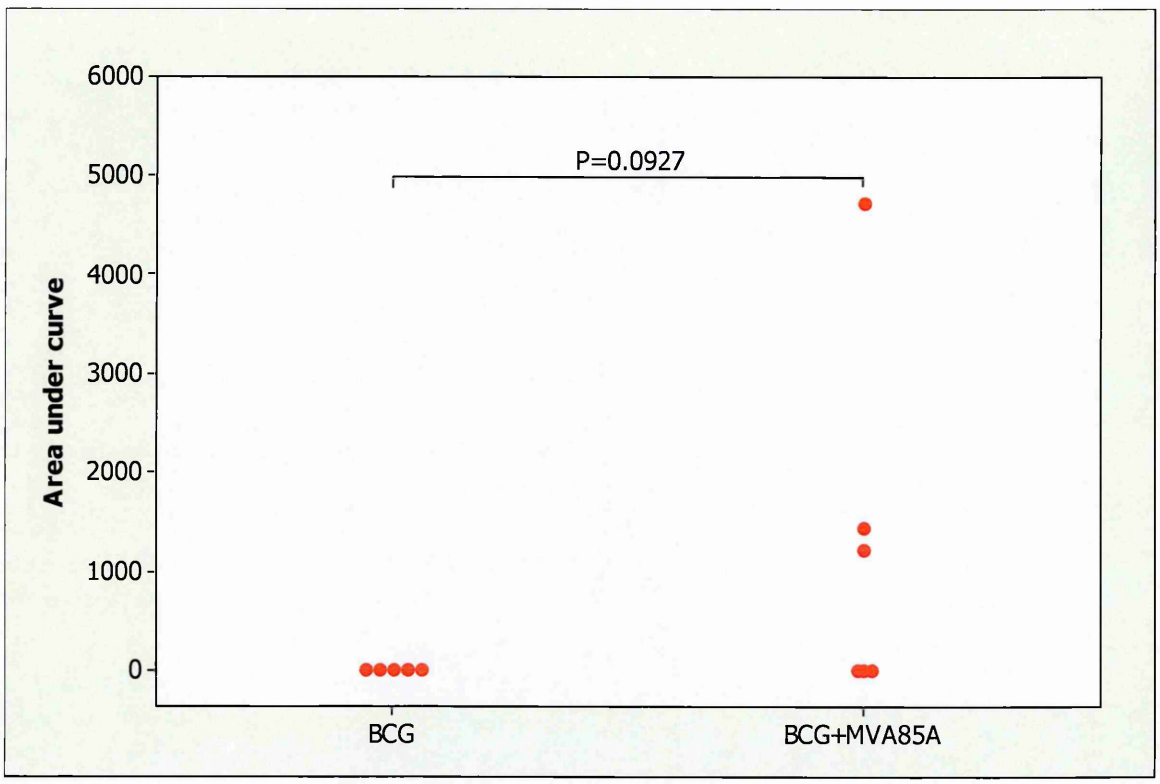


Figure 5.14: Area under the curve analysis showing differences in amount of IFN-γ secreted between BCG-vaccinated and BCG/MVA85A-immunised rhesus macaques. (Mann-Whitney statisitical test; significance, $P<0.05$).

To determine whether the levels of IFN-γ secreted in 6-day diluted whole blood supernatants were comparable to frequencies of IFN-γ-secreting cells in PBMC, results from the two assays using PPD as antigen were compared.

As shown in figure 5.15, there was a significant correlation between the two data sets ($r=0.664$, $p<0.001$) using the Spearman's rank correlation statistical test. As the frequencies of PPD-specific IFN-γ-secreting cells increased, so did the level of PPD-specific IFN-γ secretion in whole blood samples.

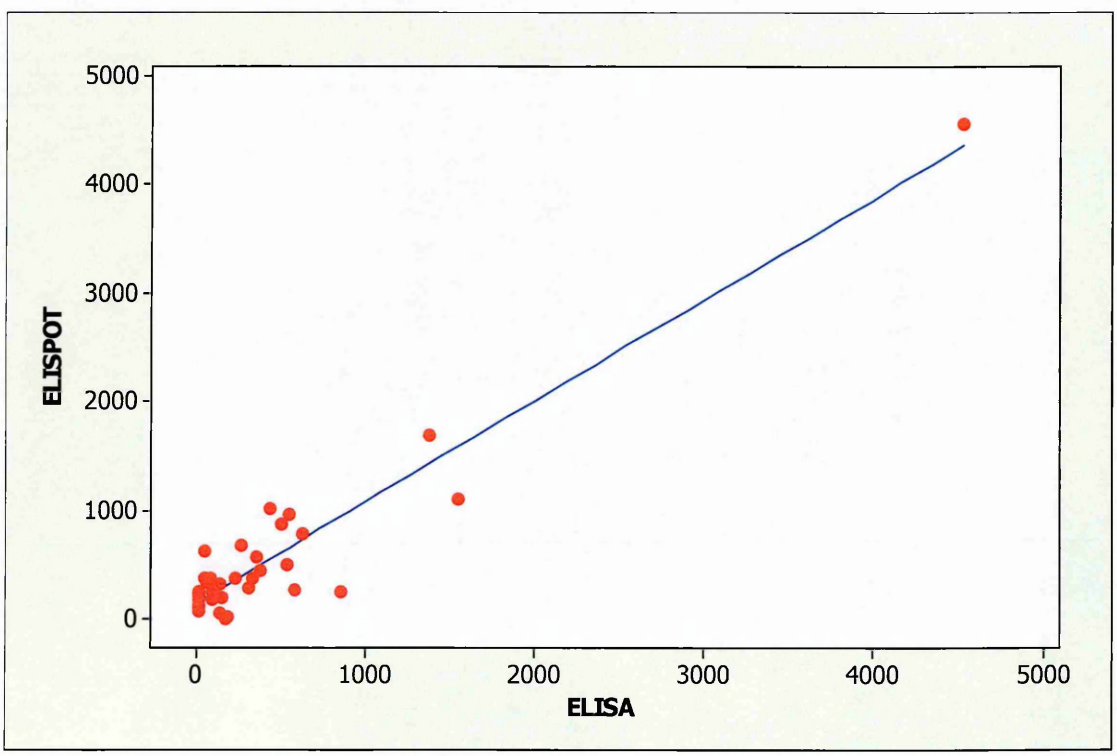


Figure 5.15: Comparison of PPD-specific responses in concentrations of IFN-γ secreted after stimulation of diluted whole blood with the frequencies of IFN-γ-secreting cells detected in PBMC after BCG/MVA85A immunisation.

5.2.2.3 Anti-vaccinia antibody responses after boosting with MVA85A

Due to not all animals showing large IFN-γ responses to PPD and Ag85A antigens, an anti-vaccinia antibody ELISA was used to determine whether animals had produced an anti-vector response. As shown in figure 5.16, all of the animals immunised with MVA85A exhibited an increase in anti-vaccinia antibody levels compared with BCG-only and unvaccinated control groups. This provided confirmation that all animals had received the MVA85A vaccine.

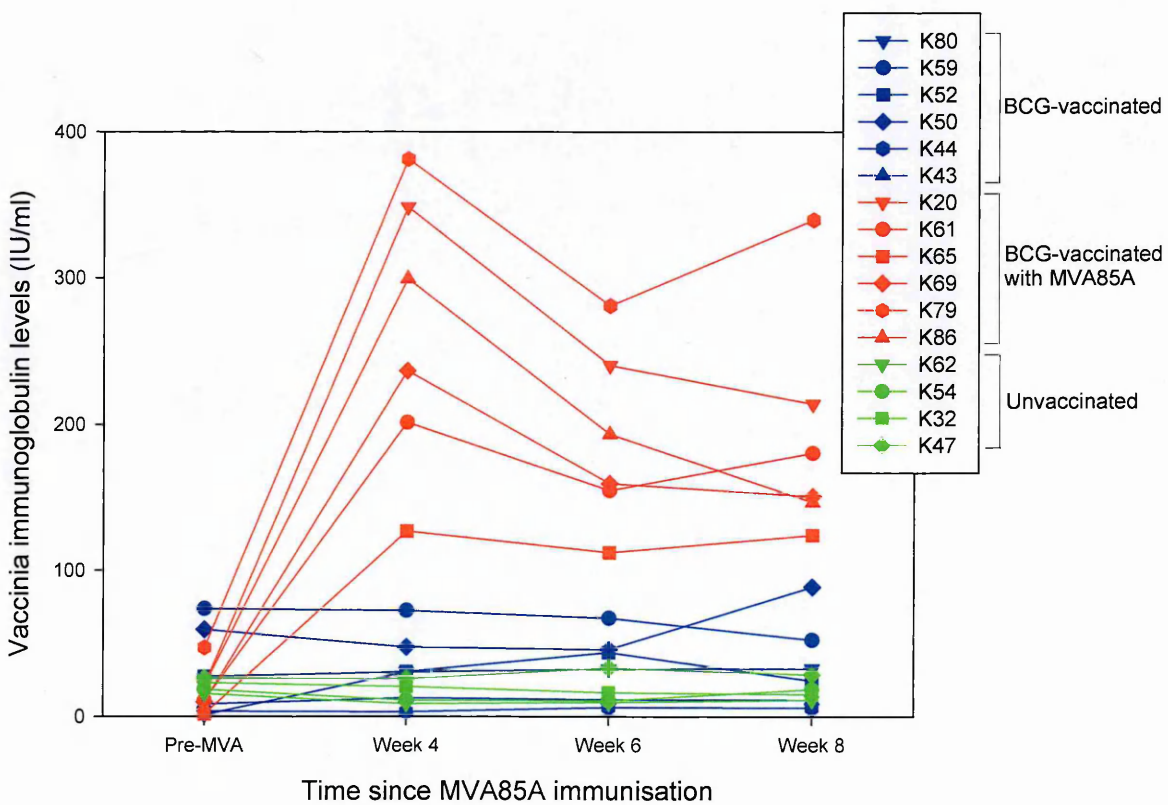


Figure 5.16: Anti-vaccinia antibody responses in rhesus macaques immunised with MVA85A compared to BCG-vaccinated only and unvaccinated controls.

5.2.3 Responses after challenge of vaccinated animals with *M. tuberculosis*

Sixteen rhesus macaques were challenged by the aerosol route with *M. tuberculosis* (estimated dose, 50 cfu), with animals divided into three groups:

- 6 animals received BCG (21 weeks prior to challenge).
- 6 animals received BCG, and 12 weeks afterwards were immunised with MVA85A (9 weeks prior to challenge).
- 4 animals were unvaccinated.

5.2.3.1 Frequencies of IFN-γ-secreting cells in vaccinated animals after challenge with *M. tuberculosis*

As with the post-vaccination phase, the frequencies of IFN-γ-secreting cells after stimulation with PPD and Ag85A peptides were measured for up to 1 year post-challenge (figure 5.17). Using PPD as antigen, responses started to rise 6 weeks after infection, whereas using Ag85A peptides the response increased in some of the animals 4 weeks post-challenge.

Using Ag85A peptide pools there was no statistical difference in response between the vaccinated and unvaccinated groups. By using PPD as antigen there were some differences between the vaccinated and unvaccinated groups of rhesus macaques when measured at individual time-points. However, when a statistical correction test was applied (Bonferroni correction) to take into account multiple comparisons, the difference became insignificant (table 5.5). Statistical analysis was only carried out during the 24 weeks after *M. tuberculosis* challenge as after this point group sizes decreased due to animals meeting humane end-points.

PPD

Mann-Whitney statistical p-value	Weeks post-*M. tuberculosis* challenge									
	1	2	4	6	8	10	12	16	20	24
BCG vs. BCG/MVA85A	**0.0051**	0.1282	0.0927	0.1282	**0.0051**	**0.0131**	**0.0122**	1.0000	1.000	0.1052
BCG vs. unvaccinated	0.1356	0.0700	0.6698	**0.0142**	0.2410	*	0.1752	0.8465	0.2472	1.0000
BCG/MVA85A vs. unvaccinated	**0.0142**	**0.0252**	0.0700	**0.0142**	0.1098	*	0.0814	0.6985	0.6985	0.2453

Ag85A peptides

Mann-Whitney statistical p-value	Weeks post-*M. tuberculosis* challenge									
	1	2	4	6	8	10	12	16	20	24
BCG vs. BCG/MVA85A	0.8102	0.9362	0.3785	0.2298	0.5752	0.0927	1.0000	1.0000	0.2472	0.4875
BCG vs. unvaccinated	0.9151	0.4555	0.6698	0.2410	0.4555	0.6171	0.5613	0.5613	1.0000	0.4875
BCG/MVA85A vs. unvaccinated	0.5224	1.0000	0.2410	0.7491	0.9151	0.4047	0.0814	0.6985	1.00000	1.0000

Table 5.5: Differences in frequencies of PPD- and Ag85A-specific IFN-γ-secreting cells after challenge with *M. tuberculosis* between vaccinated and non-vaccinated groups of rhesus macaques.

(Mann-Whitney statistical test; significance level, $P<0.05$. * = unable to perform statistical test. Light green shading indicates where individual Mann-Whitney statistical test is significant, but differences were not significant when a Bonferonni correction was applied, $P<0.005$).

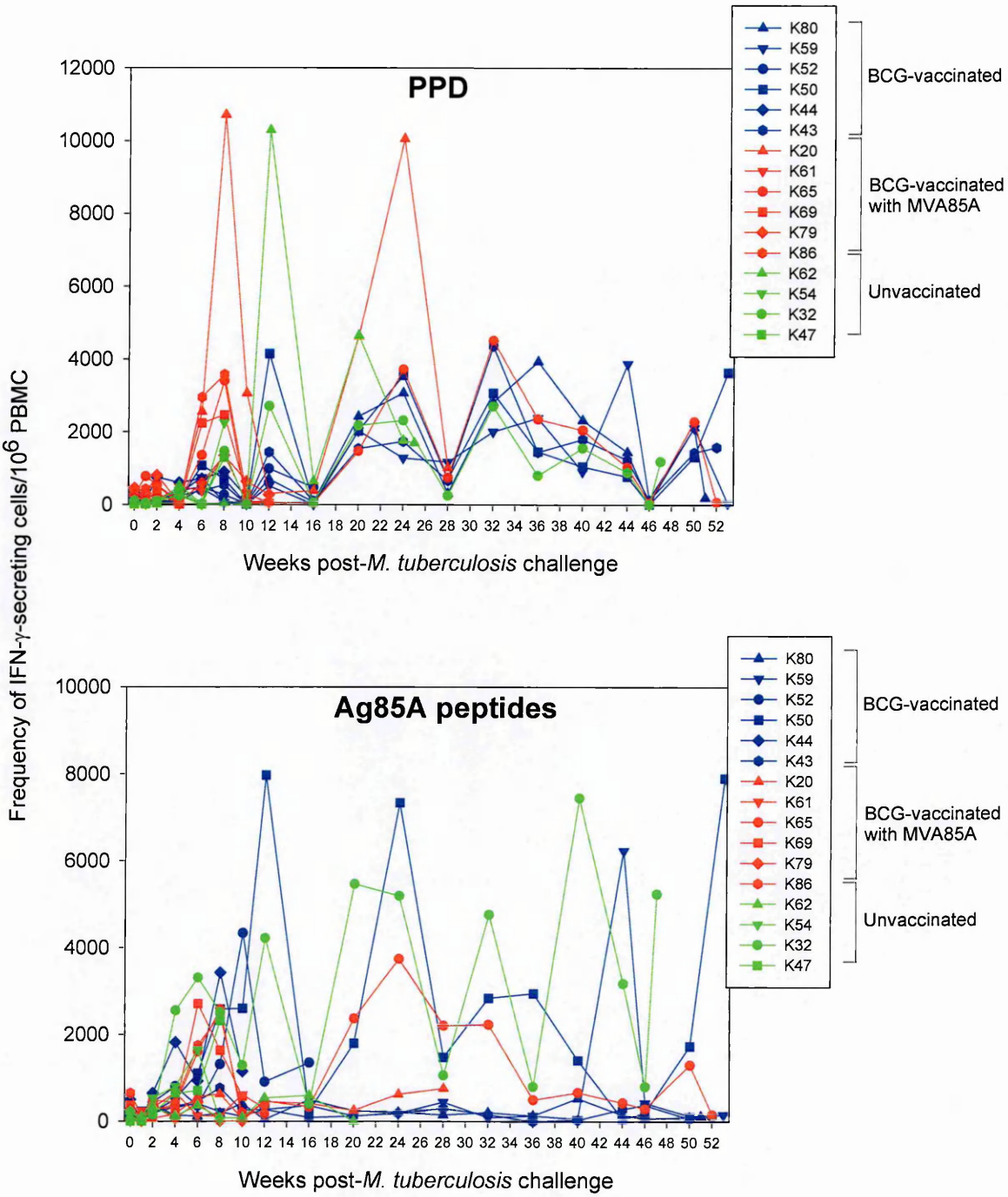


Figure 5.17: Frequencies of PPD- and Ag85A-specific IFN-γ-secreting cells after challenge of vaccinated and unvaccinated rhesus macaques with *M. tuberculosis*. (BCG-vaccinated, blue lines; BCG/MVA85A-vaccinated, red lines; and unvaccinated, green lines).

After challenge of animals with *M. tuberculosis* the responses occurred in peaks; the first beginning between 0-10 weeks post-infection and then 10-16 weeks after challenge. During the first period, the BCG/MVA85A immunised animals seemed to have the highest responses, and then during the second period the BCG-vaccinated and unvaccinated animals prevailed as frequencies of IFN-γ-secreting cells increased in these groups.

To determine whether these observations were statistically significant, areas under the curves were measured during these periods. Results confirm that 0-10 weeks post-challenge the frequencies of PPD-specific IFN-γ-secreting cells were significantly greater ($P<0.05$) in the BCG/MVA85A-immunised group than in the BCG-vaccinated or unvaccinated groups of animals (figure 5.18). 10-16 weeks post-infection the responses in the BCG/MVA85A group declined and responses in the other two groups increased, although these differences did not reach statistical significance ($P>0.05$). In contrast to the PPD-specific results, no differences were observed when Ag85A peptides were used as antigen.

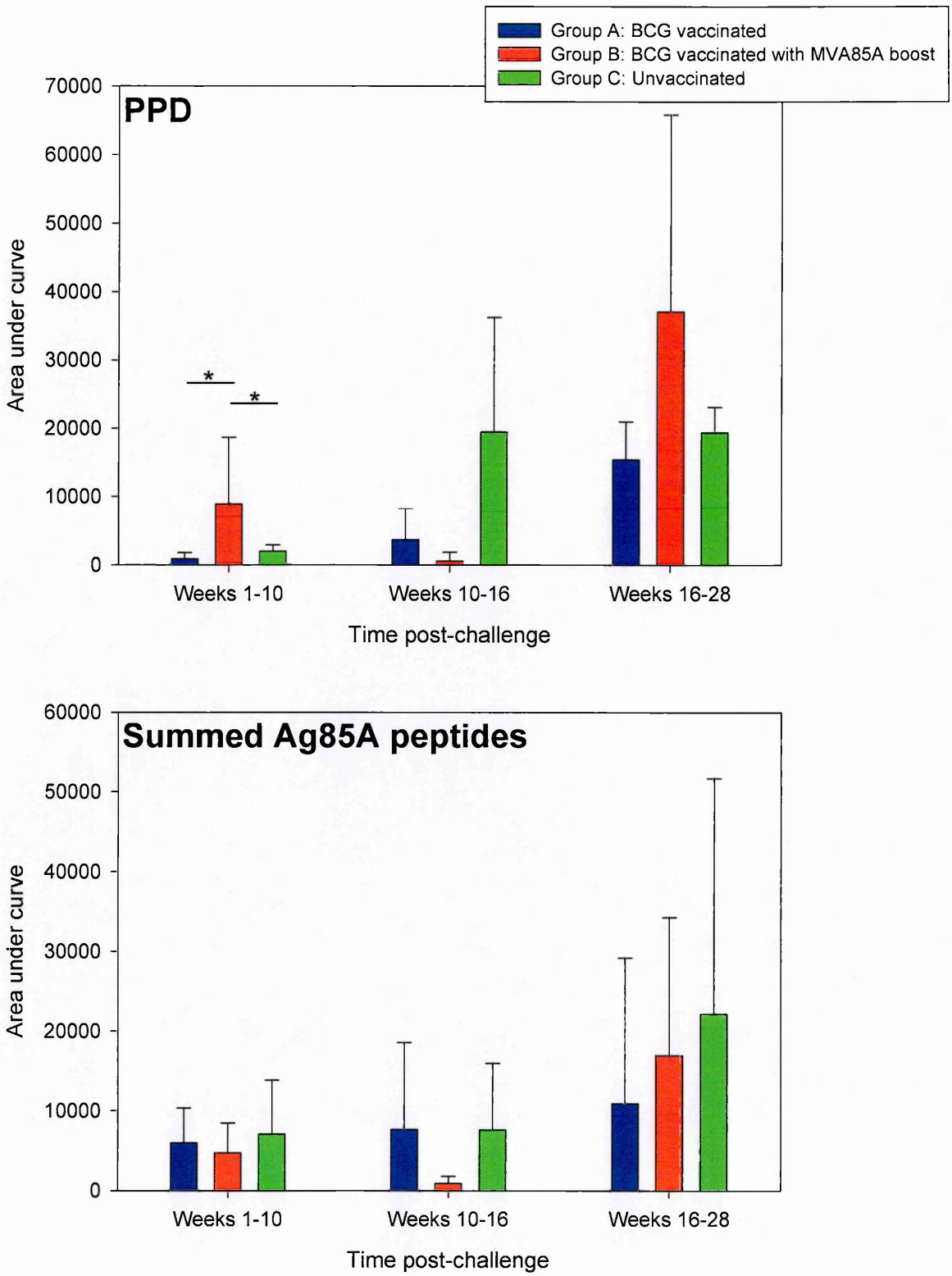


Figure 5.18: Comparison of area under the curve analysis to determine differences in frequency of IFN-γ-secreting cells at different stages post-*M. tuberculosis* challenge. (Mann-Whitney statistical test: * = P<0.05).

In addition to measuring the frequencies of IFN-γ-secreting cells after stimulation with the antigens present in the vaccine (PPD and Ag85A), *M. tuberculosis*-specific peptide antigens were also used: CFP10 and ESAT-6.

Responses to at least one of these antigens was detected in all animals 4 weeks post-challenge (figure 5.19). The kinetics of responses were similar with both antigens, with peaks at similar times and frequencies of IFN-γ-secreting cells comparable for each animal.

To determine whether the response was affected by prior vaccination, statistical analyses were undertaken at each time-point until 28 weeks post-*M. tuberculosis* challenge. At this point animal numbers had declined to such an extent that statistical analysis became less relevant. The only significant difference ($P<0.05$) observed was between the BCG and BCG/MVA85A vaccinated animals 8 weeks post-infection using CFP10 peptides to stimulate cells, where the MVA85A-immunised animals had higher responses. At all other time-points there were no differences between the groups of vaccinated and unvaccinated animals.

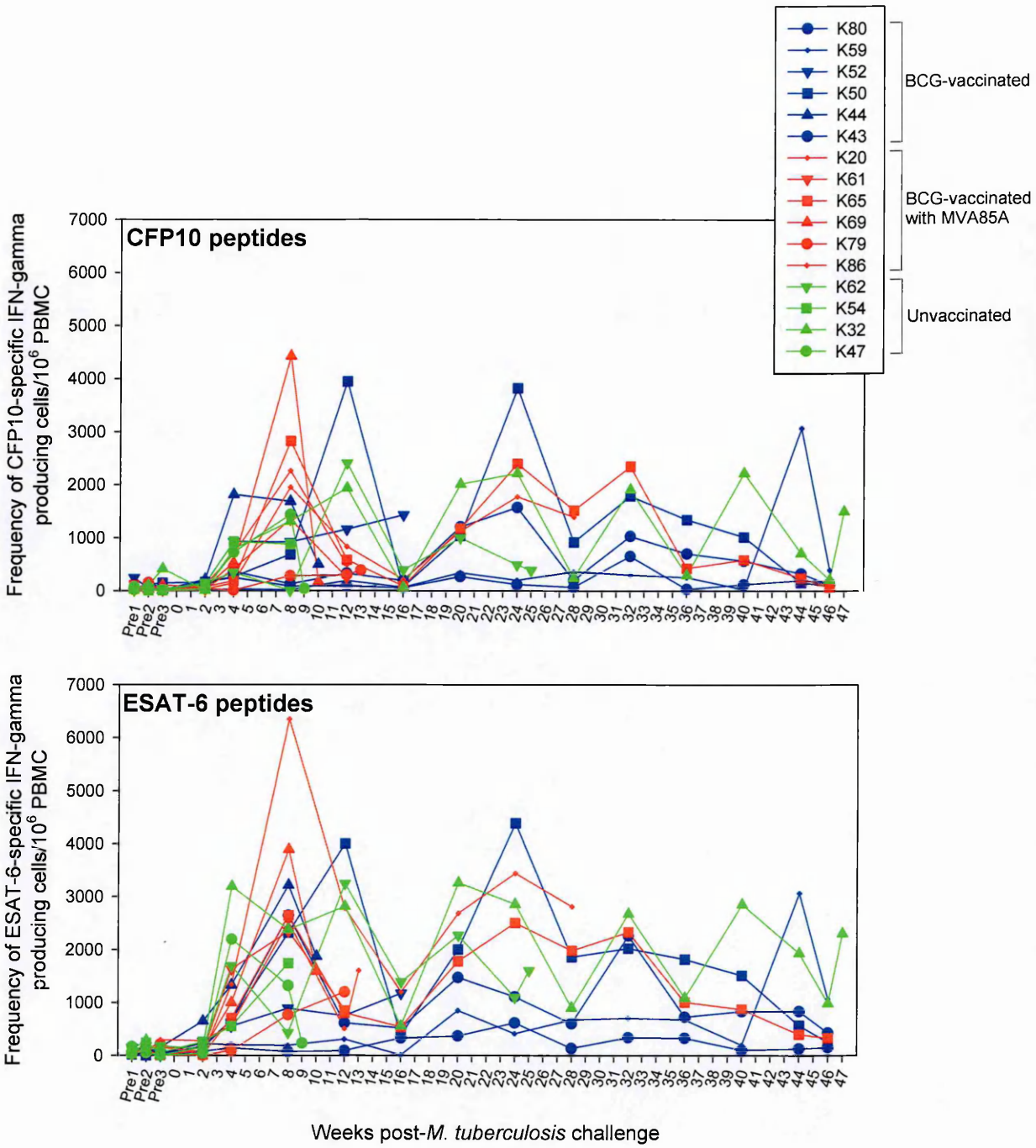


Figure 5.19: Frequencies of CFP10- and ESAT-6-specific IFN-γ-secreting cells after aerosol *M. tuberculosis* challenge of vaccinated and unvaccinated rhesus macaques. (BCG-vaccinated, blue lines; BCG-MVA85A vaccinated, red lines; and unvaccinated, green lines).

5.2.3.2 Concentration of IFN-γ secreted in vaccinated animals after challenge with *M. tuberculosis*

The amount of IFN-γ secreted in 6 day PPD-stimulated diluted whole blood samples was measured in the vaccinated and non-vaccinated rhesus macaques after infection with *M. tuberculosis*.

IFN-γ secretion was detected in all animals 4 weeks post-*M. tuberculosis* challenge (figure 5.20). Individual animals varied with the time of their peak response and the concentration of IFN-γ detected, with animal K20 showing large concentrations of IFN-γ produced at weeks 12 and 16 post-challenge.

To determine whether there were any significant differences in responses between the vaccinated and unvaccinated groups, a statistical analysis of the data was carried out (table 5.6). No significant differences ($P<0.05$) between the groups were found at any of the time-points tested.

Mann-Whitney statistical p-value	Weeks post-*M. tuberculosis* challenge								
	2	**4**	**6**	**8**	**10**	**12**	**16**	**20**	**24**
BCG vs. BCG/MVA85A	0.4712	0.6889	1.0000	0.9362	0.8102	0.1437	0.3329	0.1052	0.1052
BCG vs. unvaccinated	0.3938	0.1098	0.7491	0.5940	1.0000	0.0814	0.8465	0.8170	0.1052
BCG/MVA85A vs. unvaccinated	0.5940	0.3374	0.9151	0.5940	0.8676	1.0000	0.6985	1.0000	0.2453

Table 5.6: Statistical analysis of the differences between vaccinated and non-vaccinated groups of rhesus macaques in concentrations of PPD-specific IFN-γ secretion after challenge with *M. tuberculosis*.

(Mann-Whitney statistical test; significance level $P<0.05$).

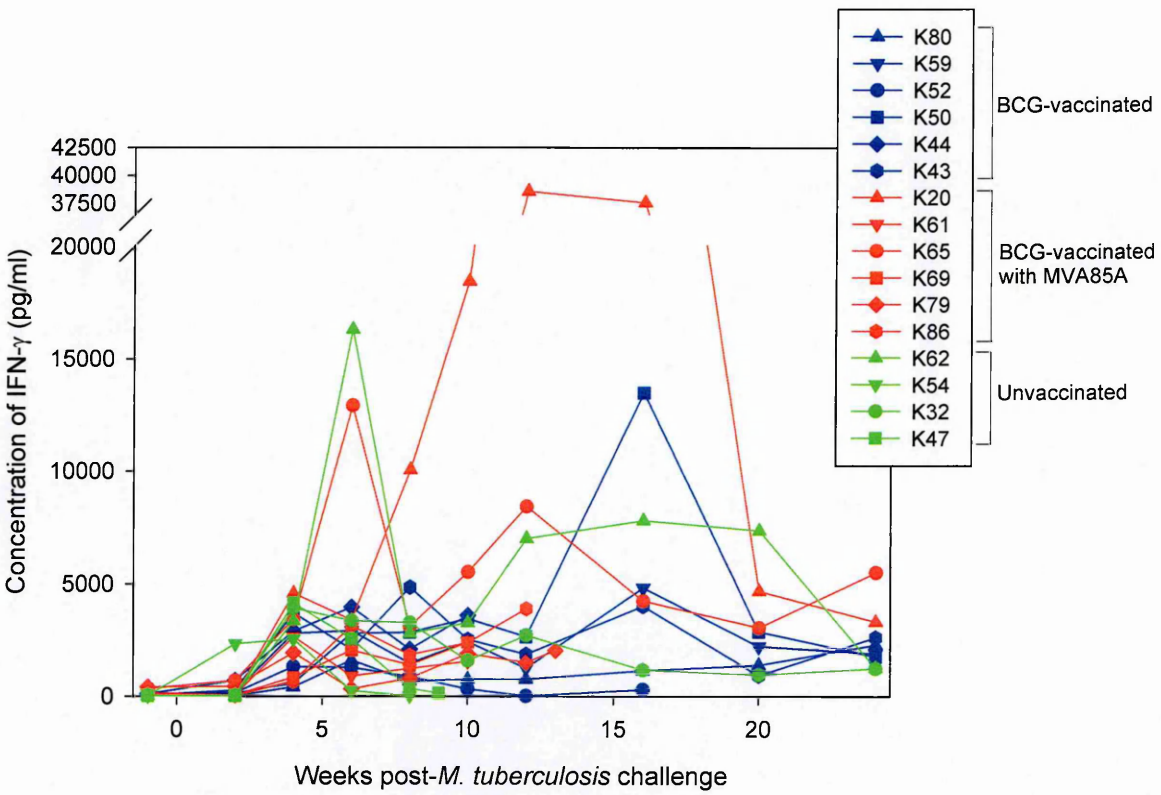


Figure 5.20: Concentrations of IFN-γ secreted in 6 day PPD-stimulated diluted whole blood supernatants in vaccinated and unvaccinated rhesus macaques after *M. tuberculosis* challenge.

(BCG-vaccinated, blue lines; BCG-MVA85A vaccinated, red lines; and unvaccinated, green lines).

As the concentration of IFN-γ secreted and frequency of IFN-γ-secreting cells after PPD stimulation were measured at the same time-points, the data were compared to see if any correlation was observed (figure 5.21). Data used covered the 24 weeks post-challenge; no correlation was seen when comparing these data sets (Spearman's rank correlation test; r_s=0.146, P=0.119). When performed on the separate vaccinated/non-vaccinated groups, no correlation was seen in the BCG-vaccinated, BCG/MVA85A-immunised or unvaccinated groups, with r_s=0.115 (P=0.435), r_s=0.187 (P=0.241) and r_s=0.131 (P=0.524), respectively.

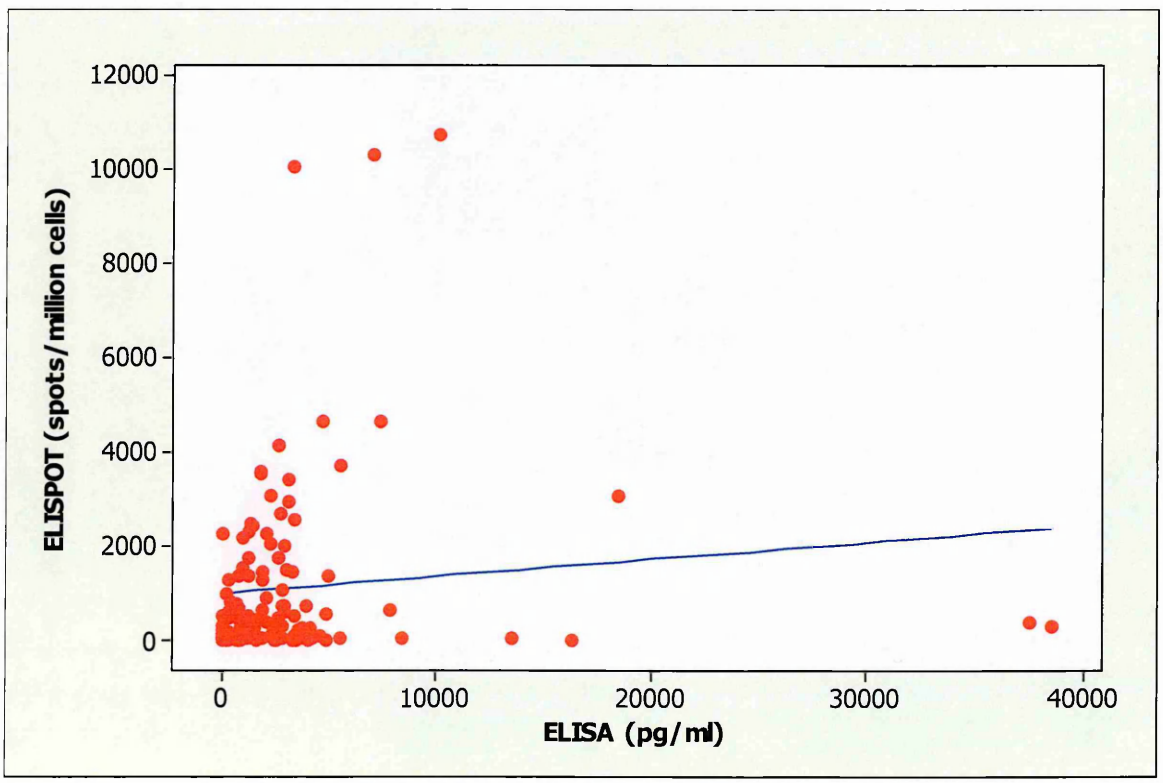


Figure 5.21: Comparison of concentration of IFN-γ secreted and frequency of IFN-γ-secreting cells after stimulation with PPD in vaccinated and unvaccinated rhesus macaques post-*M. tuberculosis* challenge.

5.2.4 Specificity of the Ag85A responses after vaccination and post-*M. tuberculosis* infection

As Ag85A peptide responses were detected after MVA85A immunisation and after *M. tuberculosis* challenge, the specificity of which peptide pools were immunogenic was studied.

Figure 5.22 shows the responses to the 7 peptide pools of Ag85A after MVA85A boosting and after *M. tuberculosis* challenge. The specificity of the response at 1 and 2 weeks after MVA85A immunisation was similar for each animal, though different between animals. Post-*M. tuberculosis* infection though, the specificity differs as the dominant pool(s) change. However, there are instances of the same peptide pool giving strong frequencies of IFN-γ-secreting cells post-MVA85A and post-challenge, such as peptide pool B in animals K20 and K79.

As the peak responses were 1 week after MVA85A immunisation, and 6 weeks post-*M. tuberculosis* infection in most animals, these time-points were looked at in greater detail. As shown in figure 5.23, the dominant responses to the peptide pools changed in most of the animals after MVA85A-boost and after *M. tuberculosis* infection. However, one animal (K79) did show similar responses, with the exception of losing the peptide pool C response after infection.

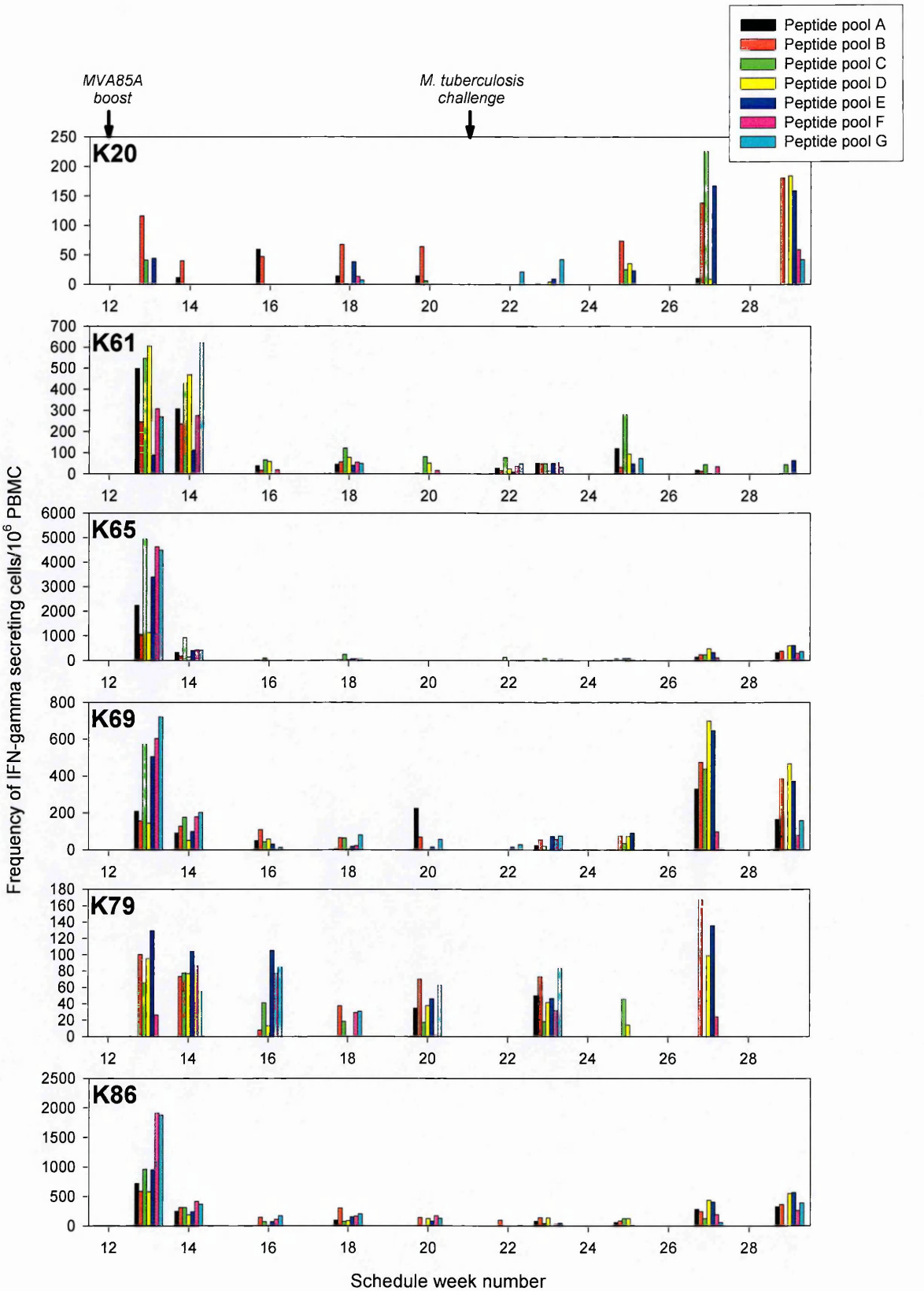


Figure 5.22: Frequencies of IFN-γ-secreting cells after stimulation with the individual Ag85A peptide pools after MVA85A immunisation and *M. tuberculosis* challenge. (MVA85A boost given at week 12; *M. tuberculosis* challenge delivered at week 21).

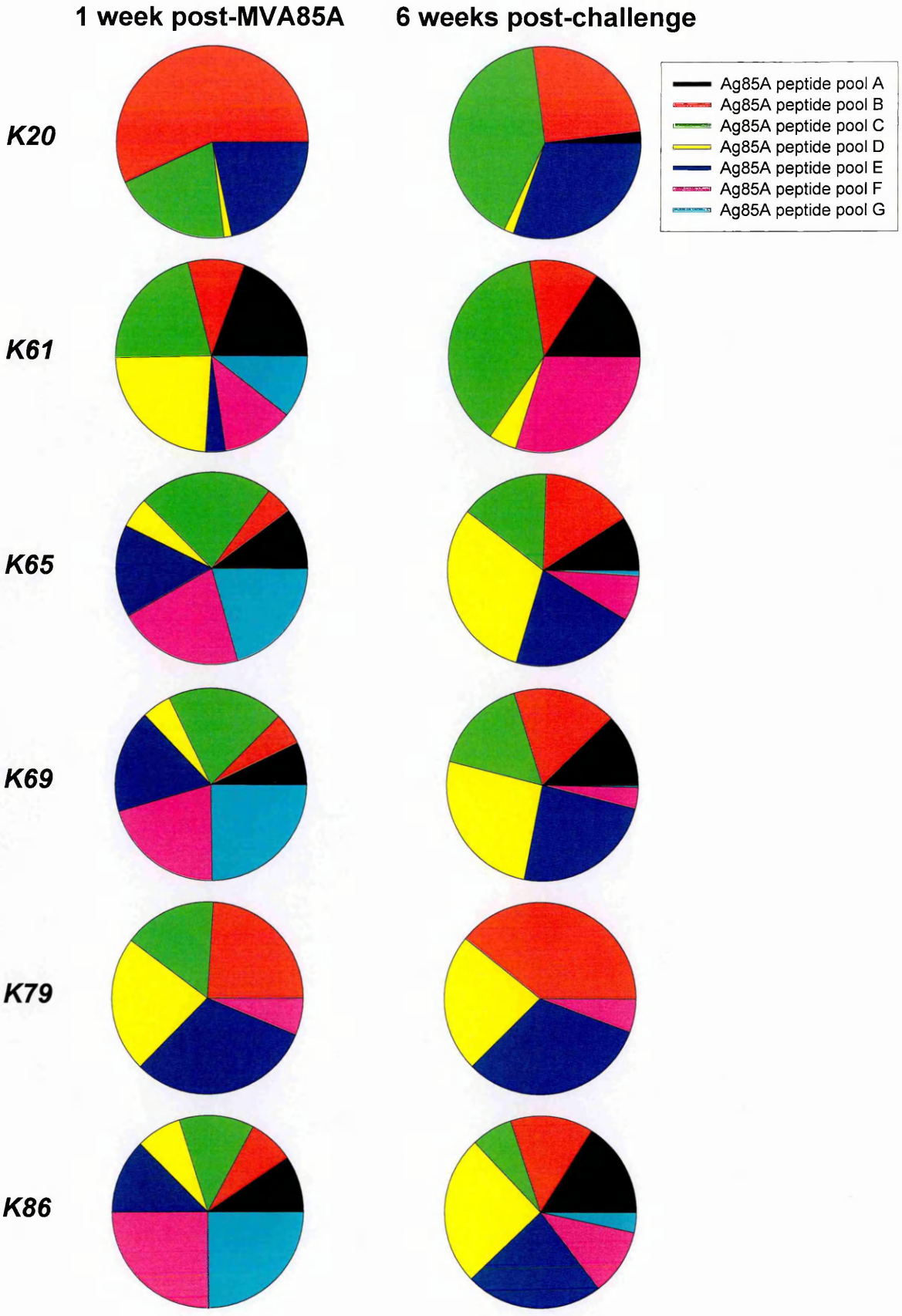


Figure 5.23: Proportion of the total Ag85A response to the individual peptide pools of Ag85A 1 week post-MVA85A immunisation and 6 weeks post-*M. tuberculosis* infection.

5.2.5 Measurements of IFN-γ and relation to survival post-*M. tuberculosis* infection

As some animals reached humane clinical endpoints prior to the end of the study, IFN-γ measurements were analysed to see if they showed any differences. Figure 5.24 shows where each animal was euthanised due to reaching humane endpoints.

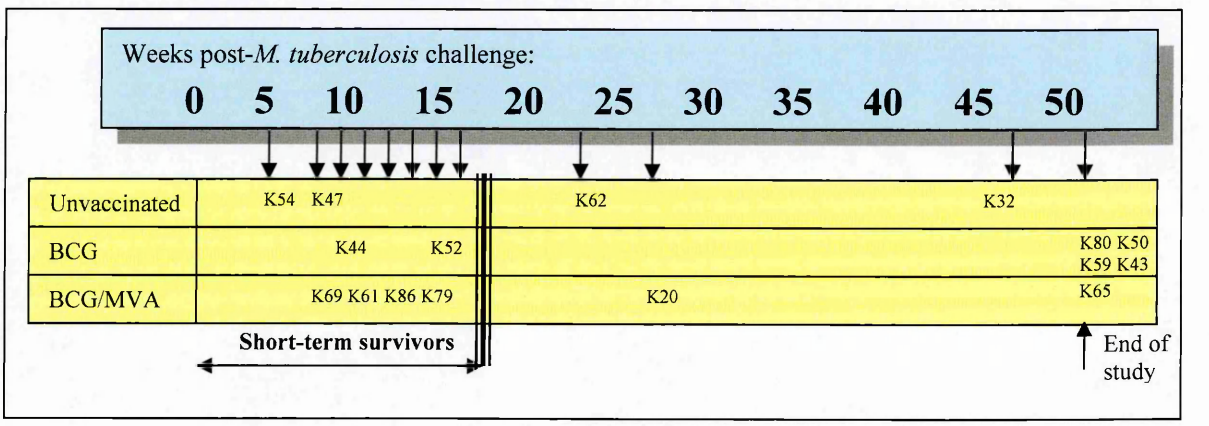


Figure 5.24: Diagrammatic overview of animals euthanised during the experimental study.

Animals were split into two groups of 8, those that were euthanised at 16 weeks or less post-challenge (short-term survivors) and those that survived for over 16 weeks (longer-term survivors), as shown in table 5.7. It was assumed that meeting the humane clinical endpoint within 16 weeks was due to more rapid disease progression in these animals.

Short-term survivors	Longer-term survivors
K54	K62
K47	K20
K44	K32
K69	K80
K61	K65
K86	K43
K52	K50
K79	K59

Table 5.7: Animals present in short-term and longer-term survival groups.

5.2.5.1 IFN-γ responses during vaccination in relation to survival post *M. tuberculosis* challenge

5.2.5.1.1 Frequency of IFN-γ-secreting cells during vaccination

To determine whether the frequency of PPD- or Ag85A-specific IFN-γ secreting cells provided a correlate of protection against *M. tuberculosis* infection, data during the vaccination period were analysed in comparison with survival times post-challenge (figure 5.25).

No trends were observed between the frequency of PPD- or Ag85A-specific IFN-γ secreting cells during vaccination and survival post-*M. tuberculosis* challenge. The results show that the BCG/MVA85A-immunised group had the highest median PPD-specific responses and survived longer. However, as this observation was made on a group consisting of only two animals error bars could not be assigned. In this group, one animal (K65) had 4555 IFN-γ secreting cells/10^6 PBMC and the other (K20) only 218; therefore a considerable range was seen between these animals.

Another way to interpret the data was to determine whether the peak response post-vaccination correlated with length of survival post-*M. tuberculosis* challenge. Using this method, it was shown that the in the BCG-vaccinated group the PPD-specific responses were significantly correlated (Pearson correlation coefficient, r=-0.922, P=0.009)) with survival time (Figure 5.26). Animals boosted with MVA85A did not show any statistical correlation between peak response and time post-infection.

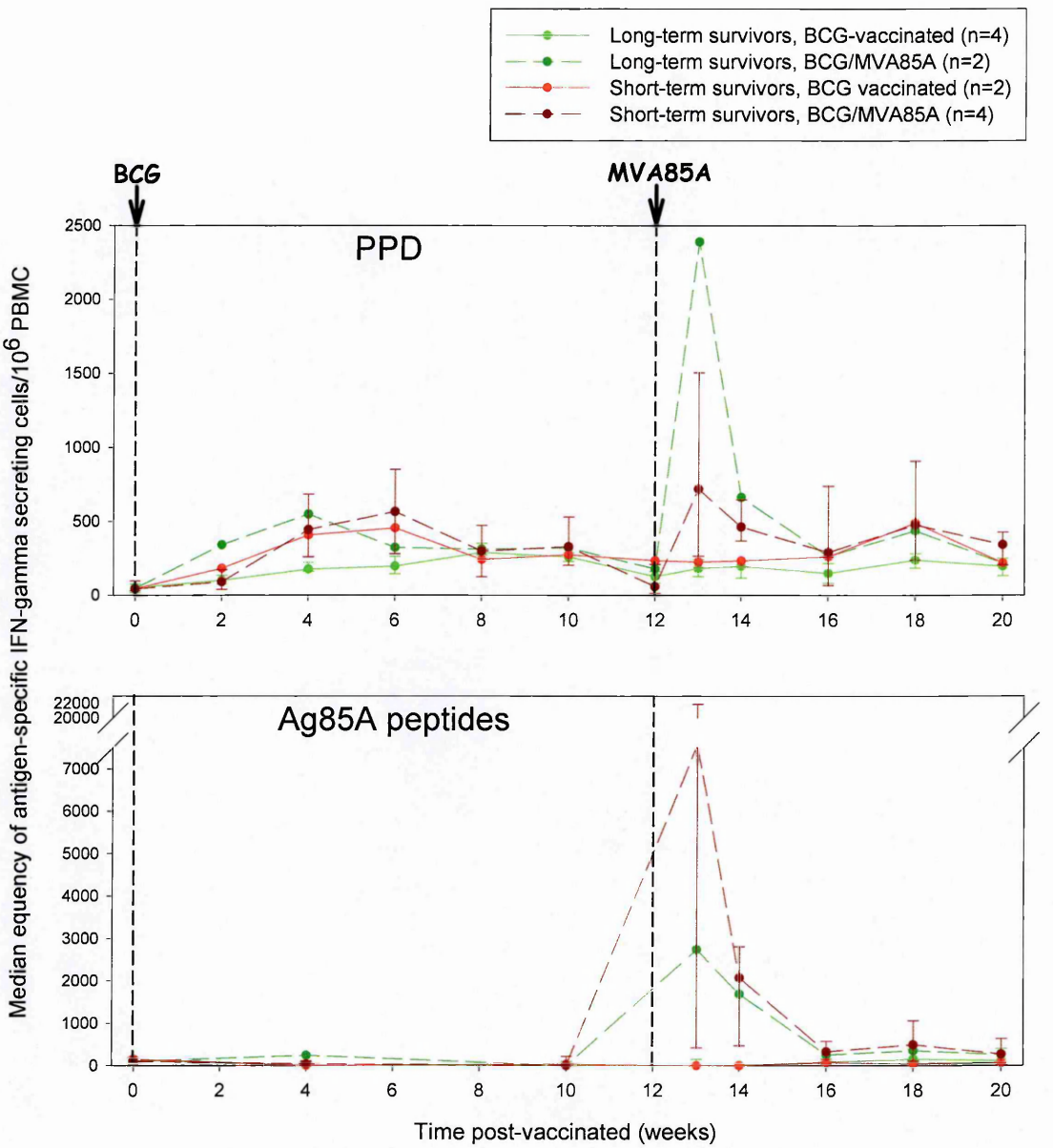
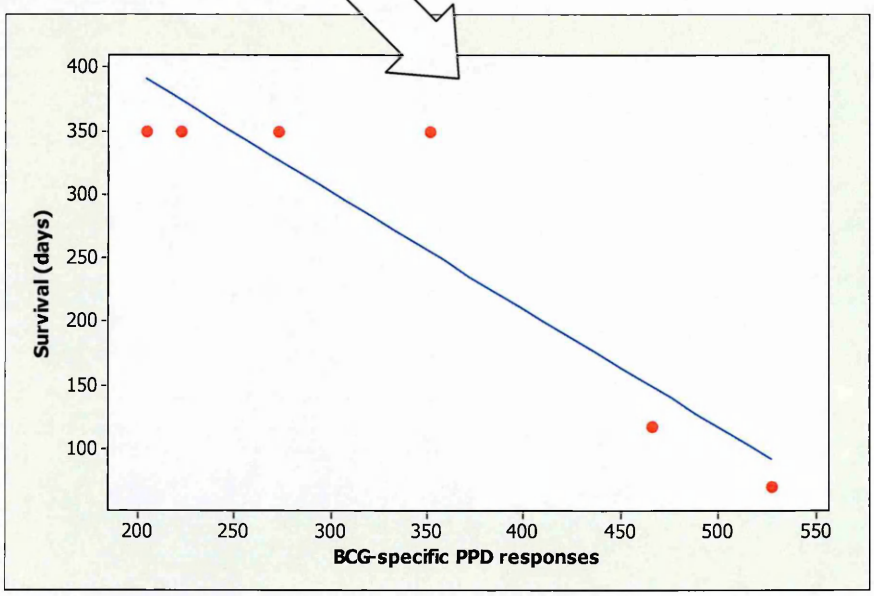


Figure 5.25: Median frequencies of IFN-γ-secreting cells after stimulation with PPD and Ag85A peptides in BCG-vaccinated and BCG/MVA85A-vaccinated rhesus macaques in relation to their period of survival after *M. tuberculosis* infection.

(Error bars denote interquartile range for group sizes consisting of 4 animals).

(a) BCG vaccination

Animal ID	Survival (days)	IFN-γ secreting cells/10^6 PBMC	
		PPD	Pearson correlation coefficient
K44	70	527	r=-0.922 (P=0.009)
K52	117	466	
K80	350	222	
K50	350	351	
K59	350	273	
K43	350	204	



(b) BCG vaccination with MVA85A boost

Animal ID	Survival (days)	IFN-γ secreting cells/10^6 PBMC			
		PPD	Pearson correlation coefficient	Ag85A peptides	Pearson correlation coefficient
K69	68	885	r=0.764 (P=0.077)	2910	r=-0.281 (P=0.590)
K61	85	368		2561	
K86	86	1642		7276	
K79	90	750		474	
K20	199	114		204	
K65	350	4320		21850	

Figure 5.26: Peak vaccination responses compared to survival post-*M. tuberculosis* challenge.

(Results displayed are values above pre-vaccination levels).

5.2.5.1.2 Concentration of IFN-γ secretion during vaccination

To determine whether the concentration of PPD-specific IFN-γ secreted in 6 day diluted whole blood supernatants related to survival, data after BCG and BCG/MVA85A vaccination were plotted (figure 5.27). Results show that the BCG/MVA85A-immunised animal with the highest secretion of IFN-γ survived for over 200 days. However, there was no overall trend with the concentration of IFN-γ post-immunisation and survival, as animals with low concentrations of IFN-γ also were in the longer survival groups.

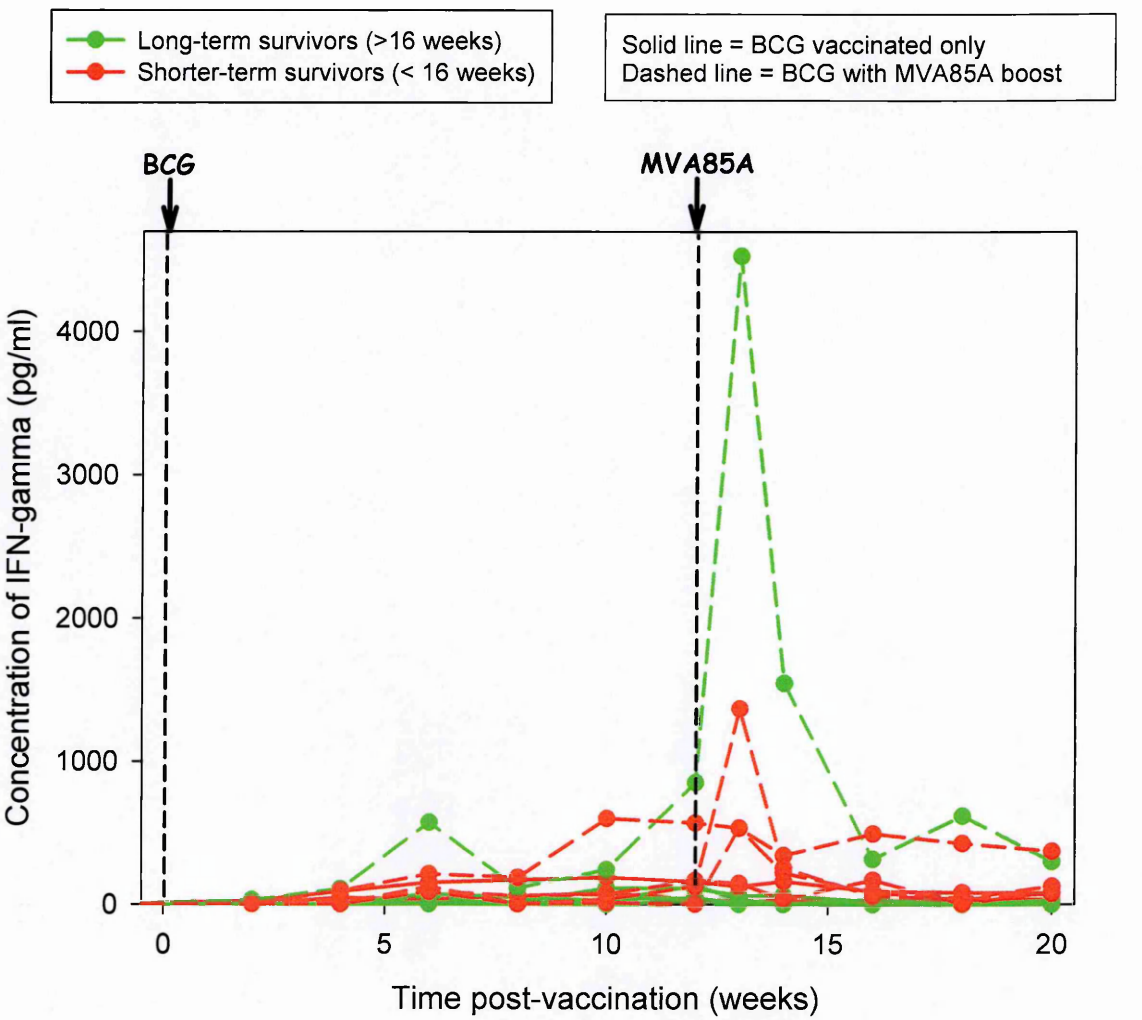


Figure 5.27: Concentration of IFN-γ secreted in diluted whole blood after stimulation with PPD for 6 days in BCG-vaccinated and BCG/MVA85A-vaccinated rhesus macaques in relation to their period of survival after *M. tuberculosis* infection.

(Long-term survivors, green lines; shorter-term survivors, red lines. BCG-vaccinated, solid lines; BCG/MVA85A-vaccinated, dashed lines).

5.2.5.2 IFN-γ responses post-challenge in relation to survival post *M. tuberculosis* challenge

5.2.5.2.1 Frequency of IFN-γ-secreting cells post-challenge

To determine whether there were any trends between the frequency of antigen-specific IFN-γ-secreting cells after *M. tuberculosis* challenge and the survival of animals, results using PPD and Ag85A were compared. As shown in figure 5.28, there was no obvious indication of a relationship between the number of cells able to secrete IFN-γ after PPD or Ag85A stimulation and survival time.

Post-challenge responses were measured against CFP10 and ESAT-6 antigens, as well as PPD and Ag85A. To determine whether there was a difference between responses to these antigens after *M. tuberculosis* infection a statistical analysis was carried out on the data in the 10 weeks following challenge. As shown in table 5.8, there were sporadic differences between responses to Ag85A, CFP10 and PPD at 2, 4 and 10 weeks post-infection, respectively. Due to no consistency in the same antigen or time-point showing a significant difference it is difficult to ascertain a definite correlation between frequency of IFN-γ-secreting cells and survival periods of animals.

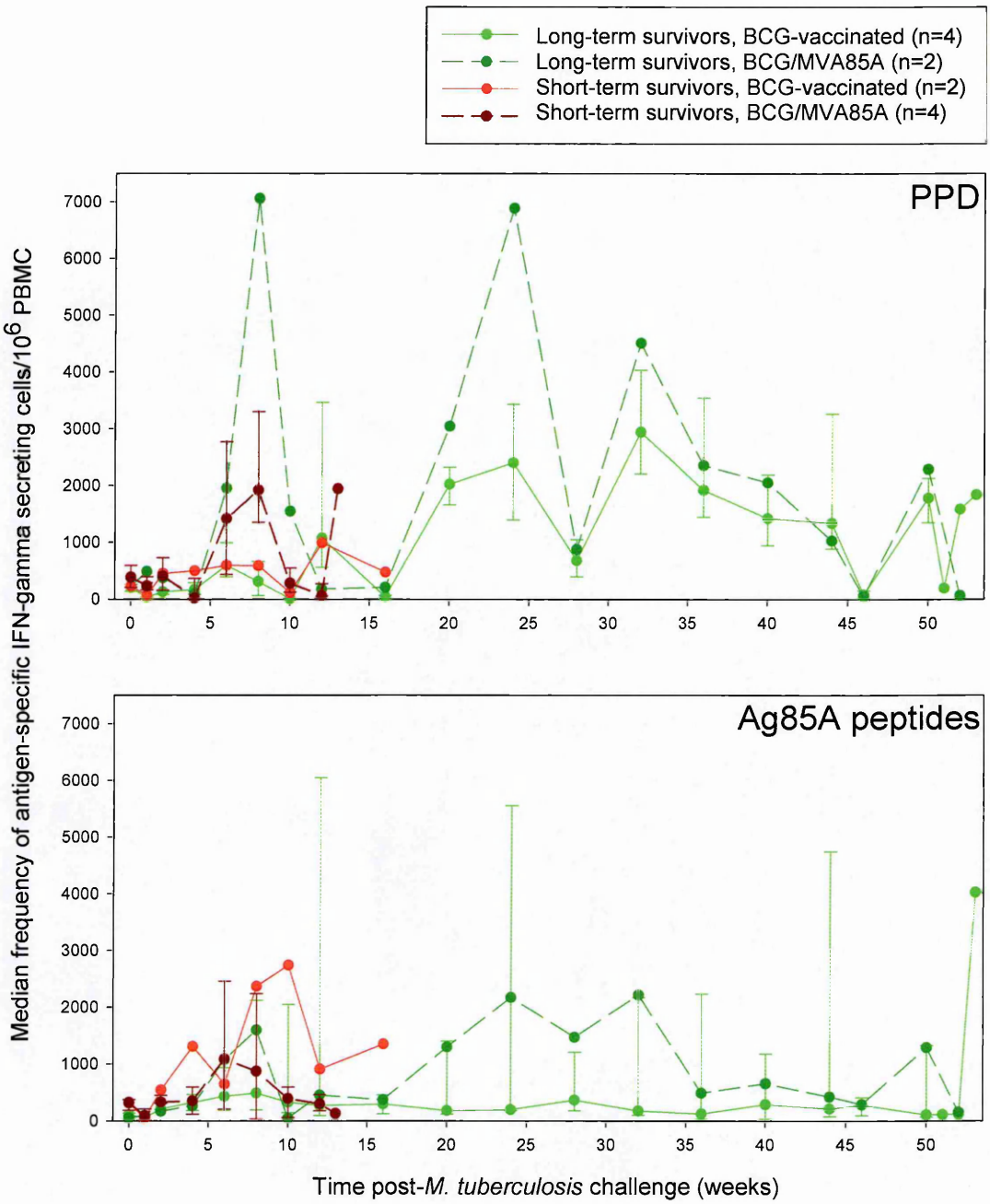


Figure 5.28: Median frequencies of PPD- and Ag85A-specific IFN-γ-secreting cells in long-term survivors (>16 weeks post-infection) and shorter-term survivors (<16 weeks post-infection).

(Error bars indicated interquartile range where groups sizes consisted of n=4).

Mann-whitney statistical p-value	Weeks post-*M. tuberculosis* challenge						
	-1	**1**	**2**	**4**	**6**	**8**	**10**
PPD	0.2131	0.7972	0.1383	0.7073	0.8279	0.0855	0.0027
Ag85A	0.3184	0.5635	0.0313	0.2271	0.7132	0.7929	0.4777
CFP10	0.0742	ND	0.8748	0.0406	ND	0.1278	ND
ESAT-6	0.2076	ND	0.4622	0.7929	ND	0.7929	ND

Table 5.8: Statistical analysis of frequencies of antigen-specific IFN-γ-secreting cells after challenge with *M. tuberculosis* in animals which survived for a short time (≤16 weeks post-infection) and those which survived longer (>16 weeks post-infection). (Mann-Whitney statistical test; significance, $P<0.05$. ND = not done).

5.2.5.2.2 Concentration of IFN-γ secreted post-challenge

To determine whether the concentration of IFN-γ secreted in 6 day PPD-stimulated diluted whole blood supernatants related to prolonged survival of animals, this measurement was compared in long-term survivors versus shorter-term survivors. As shown in figure 5.29, the animals which were euthanised ≤16 weeks post-*M. tuberculosis* had concentrations of IFN-γ less than 4200 pg/ml post-infection, whereas most of the animals which survived over 16 weeks post-challenge had greater concentrations of IFN-γ secreted.

To determine if there were any statistically significant differences in concentrations of IFN-γ secreted and prolonged survival, each time-point post-infection was analysed. As shown in figure 5.30, the difference at 6 weeks post-infection approached significance (P=0.0520). However, at 8 weeks post-infection the difference was statistically significant (P=0.0136), with animals that survived longer secreting higher concentrations of IFN-γ.

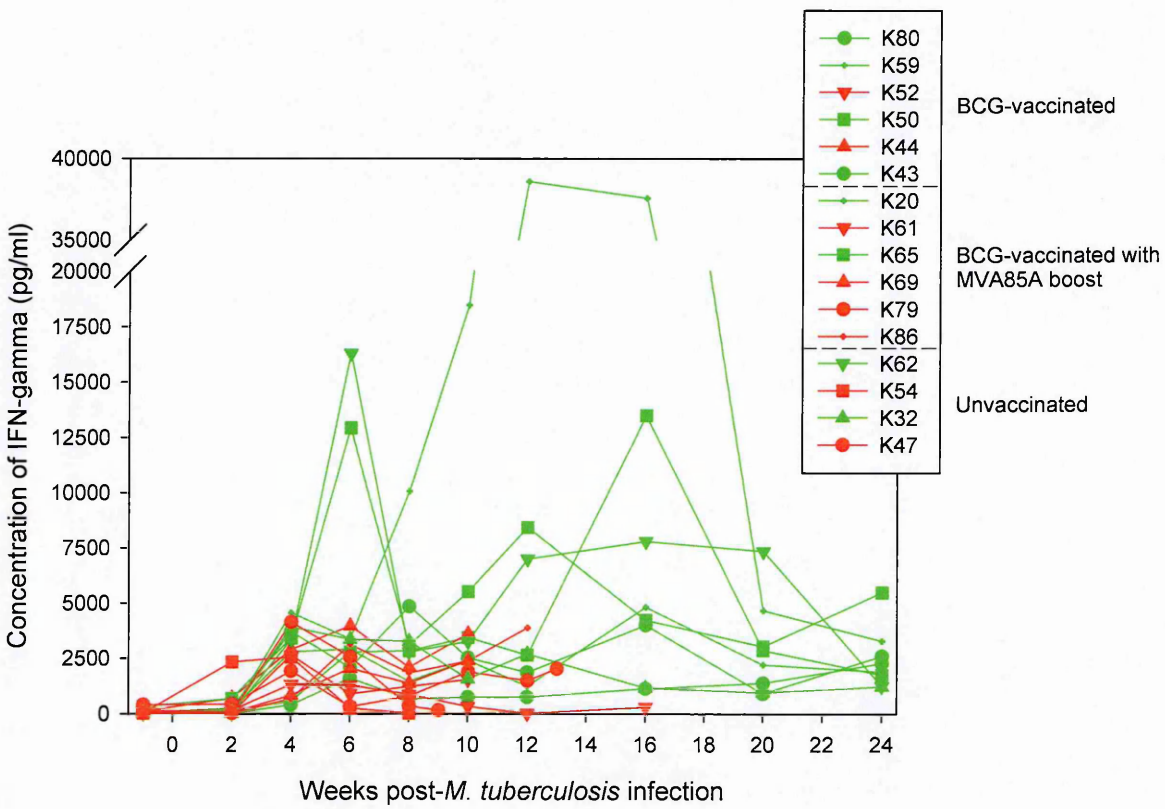


Figure 5.29: Concentration of PPD-specific IFN-γ secreted in blood from long-term survivors (>16 weeks post-infection) and shorter-term survivors (<16 weeks post-infection).

(Long-term survivors, green lines; shorter-term survivors, red lines).

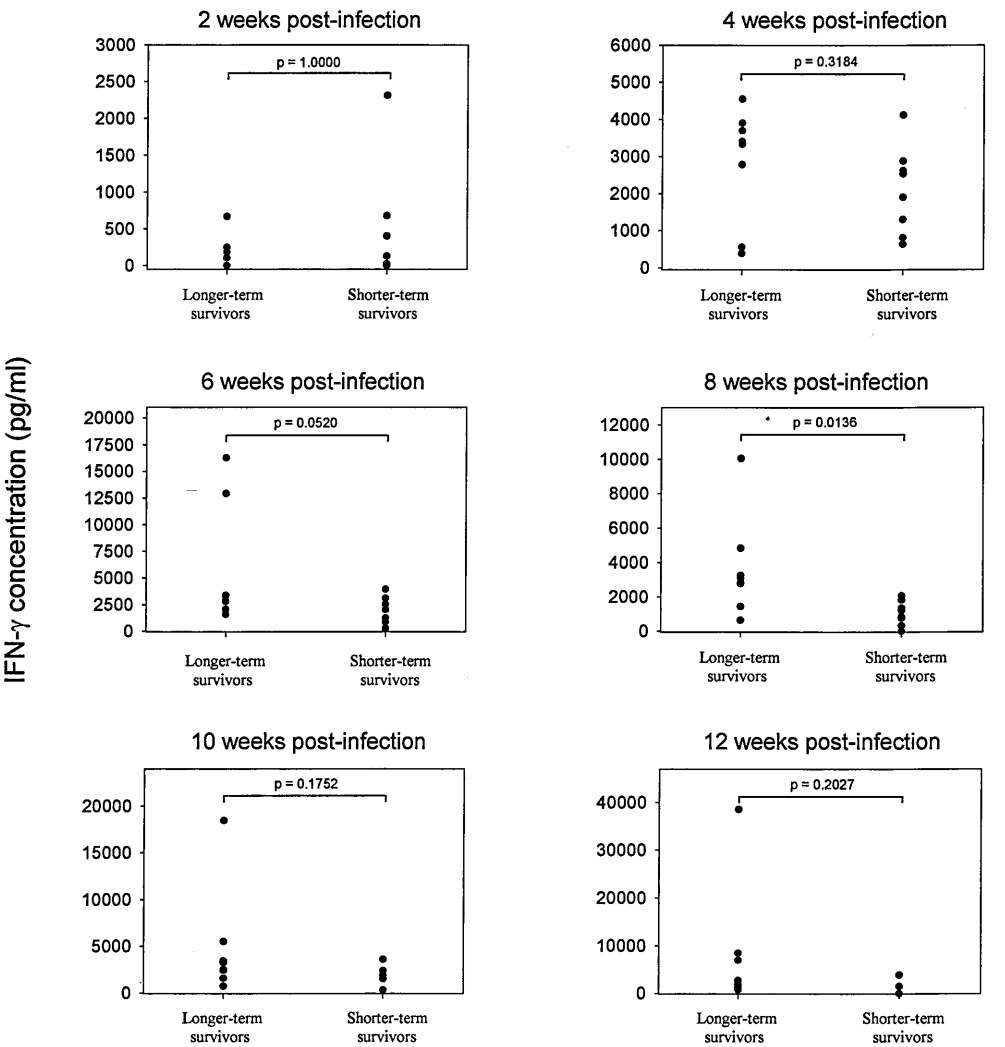


Figure 5.30: Differences in concentrations of IFN-γ secreted in 6 day PPD-stimulated diluted whole blood supernatants post-*M. tuberculosis* challenge in long-term survivors versus shorter-term survivors.

(Mann-Whitney statistical test: Significance level, $P<0.05$)

5.3 DISCUSSION

5.3.1 IFN-γ responses after BCG vaccination

5.3.1.1 Comparison of rhesus macaques and cynomolgus macaques

5.3.1.1.1 Frequency of IFN-γ secreting cells in rhesus macaques and cynomolgus macaques

In a pilot study using two rhesus macaques and two cynomolgus macaques, IFN-γ responses were monitored for over 70 weeks post-vaccination. The frequency of PPD-specific IFN-γ-secreting cells was higher in the rhesus macaques than in the cynomolgus macaques. However, given that the cynomolgus macaques only had one baseline measurement, it is difficult to determine whether the responses seen post-BCG were induced by the vaccine or represented natural fluctuations in background responses. As shown previously (figure 3.5), frequencies of PPD-reactive IFN-γ-secreting cells fluctuated by similar amounts during baseline screening of naïve animals. However, in the rhesus macaques baseline frequencies were assessed at four intervals so there could be greater confidence in the responses seen after BCG-vaccination.

Responses in macaques were detected for 70 weeks post-vaccination. In human studies where infants were BCG-vaccinated at 0-2 months of age, PPD-specific responses persisted for at least 1 year after immunisation (Marchant *et al.*, 1999). However, in other human studies, BCG-induced IFN-γ-secreting cell responses became indistinguishable from background after 24 weeks (Pathan *et al.*, 2007). These differences may be due to the latter study using subjects who were older, or due to differences in study populations with the earlier study using subjects in Africa and the latter using volunteers in the UK. In the guinea pig model, intradermal vaccination with

BCG showed that low levels of organisms persisted for at least 6 months after immunisation (Horwitz & Harth, 2003). Therefore, it could be speculated that the continued response in the rhesus macaques for over 70 weeks is due to these mycobacteria continuing to prime the immune response. However, no bacteria were detected in tissue samples collected from animals at necropsy (appendix 14, section 8.14), although this could be due to low levels or to the bacilli being at sites not measured (e.g. at the site of vaccination in the arm). Alternatively, it could be that BCG vaccination activated memory T cells that were detected long after the BCG organisms had cleared (Marchant *et al.*, 1999).

5.3.1.1.2 Concentration of IFN-γ secretion post-BCG vaccination in rhesus macaques and cynomolgus macaques

In the rhesus macaques, peak production of IFN-γ was observed 6 weeks post-vaccination. In human studies, investigators have found peak IFN-γ production 8 weeks after immunisation (Nabeshima *et al.*, 2005). However, in this study concentrations were only measured 0, 2, 4, 8, and 52 weeks post-vaccination, so the week 6 time-point was not assessed. Following BCG-vaccination, the cynomolgus macaques produced lower concentrations of IFN-γ. The observation of cynomolgus macaques producing less IFN-γ post-BCG vaccination compared to rhesus macaques has also been reported by others (Langermans *et al.*, 2001).

5.3.1.2 IFN-γ responses in a larger cohort of rhesus macaques vaccinated with BCG

The rhesus macaques were immunised with the same BCG vaccine preparation as used in the human clinical trials conducted in Oxford, thereby allowing data to be compared. This is an important factor, as it has been shown that different strains of BCG (e.g.

Japanese BCG, strain 172 and Danish BCG, strain 1331) can affect the immune response (Davids *et al.*, 2006; Wedlock *et al.*, 2007).

5.3.1.2.1 Frequency of IFN-γ secreting cells in BCG-vaccinated rhesus macaques

After PPD-stimulation, the peak frequencies of circulating IFN-γ-secreting cells were seen 4-8 weeks post-vaccination. When rhesus macaques were vaccinated with BCG intravenously and via bronchial inoculation, PPD-specific IFN-γ-producing cells were detected in PBMC 3-4 weeks post-vaccination (Lai *et al.*, 2003). Due to the different vaccination routes, it could be that delivery by intradermal injection takes longer for the immune response to be established. In parallel to the ELISPOT analysis, intracellular cytokine staining (ICS) was also carried out. However, few PPD-specific increases in $CD3^+$, $CD4^+$ or $CD8^+$ T-cell subsets were seen (appendix 15, section 8.15). This was likely due to the ELISPOT assay being more sensitive than ICS analysis.

Few responses to Ag85A peptides were observed in rhesus macaques following BCG vaccination, similar to data from human studies (McShane *et al.*, 2004; Pathan *et al.*, 2007). However, responses to Ag85A peptides have been identified in $CD8^+$ T-cells using BCG-stimulated human cells (Smith *et al.*, 2000). These responses were detected by boosting responses *ex vivo* with BCG and so measures a different parameter to that which was studied in the rhesus macaques where cell populations were not expanded.

5.3.1.2.2 Concentration of IFN-γ secreted from 6 day whole blood supernatants in BCG-vaccinated rhesus macaques

BCG-immunised rhesus macaques produced significant concentrations of IFN-γ after PPD-stimulation over the 12 weeks post-vaccination. Responses were detected at 2

weeks post-vaccination, whereas in humans no reactivity was found before 4 weeks post-vaccination (Ravn *et al.*, 1997). The human studies used stimulation of PBMC for 5 days with short-term culture filtrate; 8 weeks post-BCG vaccination the concentration of IFN-γ was 396 pg/ml in 10 non-sensitised donors (Ravn *et al.*, 1997). This is similar to the levels observed in the BCG-vaccinated rhesus macaques.

5.3.2 IFN-γ responses after MVA85A immunisation of BCG-vaccinated rhesus macaques

Of the 12 rhesus macaques vaccinated with BCG, 6 were immunised with the MVA85A vaccine. MVA85A immunisation was carried out 12 weeks after the BCG vaccination. This time-point was chosen as it needed to be long enough to allow the priming immunisation with BCG to induce antigen-specific T cells (McShane & Hill, 2005) and not interfere with the initial vaccination response induced by the BCG.

5.3.2.1 Frequency of IFN-γ-secreting cells post-MVA85A boosting

One week after MVA85A immunisation of BCG-vaccinated rhesus macaques, the frequencies of antigen-specific IFN-γ-secreting cells sharply increased, as seen in humans (Brookes *et al.*, 2008; Hawkridge *et al.*, 2008; McShane *et al.*, 2004; Pathan *et al.*, 2007). However, not all of the animals showed an increase in IFN-γ-secreting cells, a situation again similar to the human studies where one of the 17 volunteers failed to boost IFN-γ responses (McShane *et al.*, 2004).

Comparing responses of MVA85A-immunised versus BCG-only animals, there was a statistically significant difference in the frequency of PPD-specific IFN-γ-secreting cells until 2 weeks post-immunisation using PPD antigen and until 4 weeks using Ag85A peptides. In human studies, responses were statistically significantly higher in MVA85A-

boosted subjects for at least 24 weeks (McShane *et al.*, 2004) and 52 weeks (Beveridge *et al.*, 2008; Hawkridge *et al.*, 2008) following vaccination. Median responses in the boosted animals were consistently higher than their unboosted counterparts despite not reaching statistical significance.

When the MVA85A-immunised responses in the rhesus macaques were directly compared with data from human clinical trials, the median human responses were higher than the median responses seen in the rhesus macaques. Statistical analysis showed that when using PPD as antigen, there were no differences in response between rhesus macaques and humans throughout the 8 weeks post-MVA85A. With Ag85A peptides, there was no difference in the peak response 1 week post-boost, but there were differences at weeks 2, 4 and 8. The reason for the higher level of responses in humans, despite the macaques receiving a higher dose ($5x10^8$ pfu) of MVA85A compared to concentrations used in human clinical trials ($1x10^8$ pfu, Oxford trial T009), could be due to continual restimulation with environmental mycobacteria encountered by the population. It is unlikely that the rhesus macaques would encounter similar levels as air into the rooms is continually filtered, water is sterilised, and there is little contact with other subjects apart from the animal handlers. However, as the animal handlers wear personal protective equipment, this further reduces potential sources of exposure to environmental mycobacteria to the rhesus macaques.

Using ICS analysis, PPD-stimulated cells showed an increase in $CD3^+$ T cells expressing IFN-γ in animals K65 and K86 (appendix 17, section 8.17), the two animals with the highest frequency of IFN-γ-secreted cells detected by ELISPOT. When the T-cells were sub-divided, increases in IFN-γ expression were found in $CD4^+$ T-cells but not $CD8^+$. This is similar to human studies where all of the responses seen following vaccination

with BCG and MVA85A were diminished after depletion of $CD4^+$ T cells (McShane *et al.*, 2004). The lack of $CD8^+$ T-cell responses may be due to experimental design relating to the use of PPD in the ICS assay, whereas using live BCG in a restimulation assay, as used by others (Smith *et al.*, 2000), may have yielded some $CD8^+$ responses.

Using DNA vaccination with poxvirus boosting in malaria studies, the peak *ex vivo* response was at 7 days after the heterologous vaccination for each participant. When this was compared with their corresponding cultured ELISPOT response at 6 months post-vaccination, there was a significant correlation between the two assays (Keating *et al.*, 2005). This suggested that the magnitude of *ex vivo* responses has an impact on the development of a long-term memory population of T cells. Using MVA85A as a vaccine boost, if the same is true then the high frequencies of IFN-γ-secreting cells detected 1 week post-boost in the rhesus macaques may indicate the establishment of longer-term memory immunity. However, as the responses declined rapidly this may mean that there was insufficient time for optimum development of memory immune cells.

5.3.2.2 Concentration of IFN-γ secreted post-MVA85A immunisation

Secretion of IFN-γ was elevated post-MVA85A immunisation in three of the six animals following PPD stimulation – which were also the animals with the highest frequencies of PPD-specific IFN-γ-secreting cells. Increased secretion of IFN-γ has also been observed after MVA85A boosting of BCG responses in mice (Romano *et al.*, 2006). When the concentration of IFN-γ secreted was compared with the frequency of cells secreting IFN-γ after MVA85A immunisation of rhesus macaques, a significant correlation was observed. A similar correlation was not found in humans (Beveridge *et al.*, 2008).

5.3.2.3 Anti-vaccinia antibodies after MVA85A boosting

All of the MVA85A-immunised animals showed vaccinia-specific responses, whereas the BCG-only and unvaccinated animals did not show any specific increases in antibody levels. This confirmed that all animals had made an immunological response to the vector; so although not all animals showed strong IFN-γ responses, this was not due to failure of vector delivery. However, as insert-specific responses were not measured they could have been a failure in antigen 85A presentation to the immune response.

5.3.3 IFN-γ responses after *M. tuberculosis* challenge of vaccinated rhesus macaques

5.3.3.1 Frequency of IFN-γ-secreting cells after *M. tuberculosis* challenge of vaccinated animals

The increase in frequencies of PPD-specific IFN-γ-secreting cells occurred sooner after *M. tuberculosis* infection in the BCG/MVA85A-immunised rhesus macaques than either the BCG-vaccinated animals or the unvaccinated group. In BCG-vaccinated rhesus macaques, the ability of $CD4^+$, $CD8^+$ and γδ T cells to mount faster and stronger recall responses were considered to be important mechanisms underlying the immune protection against mycobacterial infection (Lai *et al.*, 2003). In mice vaccinated with BCG and protected from *M. tuberculosis* infection, an early increase at 2 weeks post-challenge of IFN-γ in lungs compared to naïve animals was seen (Goter-Robinson *et al.*, 2006; Rodgers *et al.*, 2006). These differences diminished as the naïve mice mounted their own immune response to infection. The early vaccine-induced cytokine responses in immunised animals limited bacterial growth during the early stages of infection which resulted in a decreased bacterial burden during the chronic stage of the infection (Goter-Robinson *et al.*, 2006). Therefore, by decreasing bacterial growth during early stages of

infection, these vaccine-induced cytokine responses to mycobacteria may enhance survival of vaccinated animals .

Increases in IFN-γ^+ T-cell populations ($CD3^+$, $CD4^+$ and $CD8^+$) measured by intracellular cytokine staining showed that most of the responses post-*M. tuberculosis* challenge were $CD4^+$-specific (appendix 18, section 8.18). However, antigen-specific $CD8^+$ T cells were identified in 2 MVA85A-boosted animals (K20 and K86). This may indicate that MVA85A enhances the cytotoxic T-lymphocyte (CTL) response in some of the poxvirus-boosted animals, although further work would be needed to confirm this.

5.3.3.2 Concentration of IFN-γ secreted after *M. tuberculosis* challenge of vaccinated animals

There were no significant differences in concentrations of IFN-γ secreted between the vaccinated groups of animals and the unvaccinated group. This observation has also been reported in mice, where BCG-vaccinated and unvaccinated animals produced similar concentrations of IFN-γ post-infection (Elias *et al.*, 2005), even though the BCG-vaccinated group had some protection from disease. In human studies, despite differences in initial IFN-γ levels in BCG-vaccinated and unvaccinated household contacts, after becoming infected with *M. tuberculosis* final levels of IFN-γ were also similar (Whalen *et al.*, 2006).

5.3.3.3 Specificity of the peptide responses during vaccination and after *M. tuberculosis* infection

To see if the same peptides reacted after MVA85A-immunisation and after *M. tuberculosis* challenge, individual responses to the Ag85A peptide pools were analysed.

In 5/6 animals the response varied between those seen after MVA85A-immunisation and those detected after infection, showing that the specificity of response changed.

After MVA85A-immunisation of rhesus macaques, the response detected to the peptide pools was similar in specificity for each animal at each of the time-points. However, each individual macaque showed its own immunodominant peptide pool recognition, with different pools being immunogenic in different animals. This is similar to humans where, after MVA85A-boosting, mapping the specificity of the response to Ag85A by IFN-γ ELISPOT revealed that different epitope targeting patterns were observed in each of the 2 subjects tested (Beveridge *et al.*, 2007). Also similar to the rhesus macaques, the responses in humans decreased but the epitope specificity displayed at week 1 post-MVA85A boosting was maintained at week 24 (Beveridge *et al.*, 2007).

5.3.4 IFN-γ as a correlate of protection

5.3.4.1 Survival of vaccine groups

Throughout the analysis so far, differences in IFN-γ responses have been analysed in light of the vaccination protocols for the groups of animals. Importantly, BCG-vaccinated animals were found to be significantly protected from *M. tuberculosis* infection compared to unvaccinated controls (appendix 19, section 8.19). This is in line with earlier studies (Anacker *et al.*, 1972; Barclay *et al.*, 1970; Barclay *et al.*, 1973; Good, 1968; Janicki *et al.*, 1973; Ribi *et al.*, 1971; Schmidt, 1972) and more recent work where BCG-vaccinated rhesus macaques remained clinically healthy during a 2.5-month period of follow-up after *M. tuberculosis* challenge (Shen *et al.*, 2002). However, a study comparing BCG vaccination efficacy in rhesus macaques and cynomolgus macaques found no significant protection in rhesus macaques (Langermans et al., 2001). This difference may be due to a large dose of 3,000 cfu *M. tuberculosis* being delivered via

intratracheal instillation in the latter study compared to 50 cfu delivered by aerosol in this study. Also, in the Langermans *et al* study, BCG was removed with chemotherapy after 8 weeks (Langermans *et al.*, 2001). As BCG is known to persist for over 6 months in guinea pigs (Horwitz & Harth, 2003), perhaps there was insufficient priming of the immune response or that a continual BCG presence was required for protection.

Using the rhesus macaque model, MVA85A immunisation abolished protection above that of BCG alone from *M. tuberculosis* infection in this study (appendix 19, section 8.19). This is in contrast to another study in rhesus macaques performed at the Biomedical Primate Research Centre (BPRC), The Netherlands (Verreck-FAW *et al.*, "New primary and booster vaccination strategies display significant protection in a rhesus monkey model for tuberculosis", poster presented at Tuberculosis: from lab research to field trials conference, 2007). In the BPRC study, BCG/MVA85A-vaccinated animals showed protection by overall pathology scores, lung pathology, chest X-ray scores at autopsy, body weight and C-reactive protein levels. Although this thesis reports a different measure of outcome, i.e. survival, the BPRC study lasted for up to 18 weeks post-challenge and no BCG/MVA85A-immunised animals were reported to have reached clinical end-points. As 4 of the 6 BCG/MVA85A-immunised animals in the studies described in this thesis reached end-points before 18 weeks, it is apparent that animals in the BPRC study survived longer. The differences in this study include the period between BCG and MVA85A boost (9 weeks compared to 12 weeks in this thesis) and a difference in challenge (1,000 cfu delivered by intratracheal instillation compared to 50 cfu delivered by aerosol in this thesis). It could be speculated that housing conditions of the primates varied; for example, both colonies allow their macaques outdoors during certain periods prior to experimental study, which may mean they are exposed to different strains and levels of environmental mycobacteria. Alternatively, it may be that differences in animal genetics contributed to the differences in response. One

comparison between studies would be to look at differences in IFN-γ responses, especially 1 week post-MVA85A boost. However, in the BPRC study the assay failed at this timepoint (Verreck-FAW, personal communication) preventing this comparison from being made.

Protection against *M. tuberculosis* using MVA85A boosting of BCG vaccination has showed mixed results in mice. A decrease in bacterial load in the spleen and lung 6 weeks after aerosol *M. tuberculosis* challenge has been observed (Goonetilleke *et al.*, 2003). Others, however, have shown that MVA85A did not result in a significant enhancement in survival as boosted animals had a median survival time of 18.5 weeks compared to 19 weeks when given BCG alone (Romano *et al.*, 2006). As well as using different outcomes to measure success, the route of *M. tuberculosis* infection was i.v. in the latter study compared to aerosol delivery in the former, thus likely contributing to differences seen between the two studies.

In the guinea pig model, protection was seen after poxvirus boosting of BCG responses (Williams *et al.*, 2005a). The differences between the guinea pig studies showing protection and the study in mice (Romano *et al.*, 2006) and rhesus macaques that did not show protection could be due to several factors. Firstly, the guinea pig studies included a boost with Ag85A contained within a fowlpox virus vector (FP85A) in addition to MVA85A (Williams *et al.*, 2005a). Also, there were differences in time between BCG vaccination and MVA85A boosting: 9 weeks in the rhesus macaques, 8 months in the mouse study (Romano *et al.*, 2006), and 1 month in the guinea pig experiments (Williams *et al.*, 2005a). However, in humans a difference in boosting interval did not significantly affect the peak vaccine immune responses (Pathan *et al.*, 2007). Challenge routes also varied, with intravenous challenge being used in the mice (Romano *et al.*, 2006) but aerosol infection in the rhesus macaques and guinea pigs (Williams *et al.*,

2005a). It has also been hypothesised that after boosting, the cytokine secretion pattern in guinea pigs may be different due to immunological differences between animal species (Wakeham *et al.*, 1998). Recent advances in cytokine work in guinea pigs, including the analysis of cytokine mRNA profiles in-situ (Ly *et al.*, 2008) and the development of monoclonal antibodies for IFN-γ (Schafer *et al.*, 2007), will allow this hypothesis to be tested out in the future.

5.3.4.2 Survival and post-*M. tuberculosis* infection responses

5.3.4.2.1 Frequency of IFN-γ-secreting cells and rapid disease progression of animals during M. tuberculosis infection

No consistent difference could be found between those animals that survived >16 weeks post-infection and those that did not in terms of the frequency of circulating antigen-specific IFN-γ-secreting cells post *M. tuberculosis* challenge. This has also been recently reported in the mouse model of BCG-vaccination and *M. tuberculosis* infection, where frequencies of Ag85A-specific IFN-γ-secreting T cells did not correlate with protection, but instead reflected the load of *M. tuberculosis* infection (Mittrucker *et al.*, 2007).

5.3.4.2.2 Concentration of IFN-γ secreted and rapid disease progression of animals during M. tuberculosis infection

Data from experimental animals and patients with pulmonary tuberculosis and their household contacts indicate that *M. tuberculosis*-stimulated whole-blood production of IFN-γ, although imperfect, is the best available correlate of protection (Ellner *et al.*, 2000; Kaufmann, 2006b).

When the concentration of IFN-γ was measured in PPD-stimulated diluted whole blood supernatants, differences were observed between 6-8 weeks post-infection. In the animals that survived >16 weeks post-*M. tuberculosis* challenge, significantly higher concentrations of IFN-γ were secreted. However, the significance disappeared when only vaccinated animals were analysed (appendix 20, section 8.20). Therefore, the ability of rhesus macaques to secrete IFN-γ after *M. tuberculosis* infection indicates a good prognosis, but does not correlate with vaccine-induced protection. Others have shown that the magnitude of the BCG-attributable increase in IFN-γ responsiveness to PPD, from before vaccination to 1 year after vaccination, correlates better with the known levels of protection induced by immunisation with BCG than does the absolute value of the IFN-γ response (Black *et al.*, 2002). The study described in humans (Black *et al.*, 2002) measured responses in populations using large numbers of individuals (n=613), whereas responses in macaques used in this thesis consisted of smaller group sizes and responses were determined on an individual animal basis. Also, as the majority of the rhesus macaques had no IFN-γ detected in supernatants prior to vaccination, a similar analysis could not be undertaken in the macaque model.

The finding in the rhesus macaques that the concentration of IFN-γ secreted in PPD-stimulated whole blood supernatants 6-8 weeks post-challenge correlated with an increased survival time could be due to the 6-day incubation period of this assay, rather than the overnight stimulation of the ELISPOT assay. This extended period may have allowed memory responses to be differentiated from effector responses.

5.4 CONCLUSION

When analysed alongside data generated from human clinical trials, it has been found that IFN-γ responses after BCG-vaccination are comparable with data in rhesus macaques. Using a novel 'booster' vaccine of MVA85A, kinetics of responses were similar but magnitudes of IFN-γ secreting cells were lower in rhesus macaques compared to humans, despite a higher vaccine dose being administered. Vaccinated animals were challenged via aerosol with *M. tuberculosis*, with this being the first study where IFN-γ measurements have been undertaken throughout such a time-course. It was found that BCG-vaccinated rhesus macaques were significantly protected against disease as measured by survival against aerosol *M. tuberculosis* challenge, but MVA85A-boosted animals were not. In the animals that showed signs of protection there were no differences in IFN-γ responses compared to those that were not protected.

6 GENERAL DISCUSSION

6.1 ANSWERING THE HYPOTHESIS

The hypothesis this thesis set out to test was:

"IFN-γ measurements in a non-human primate experimental model of tuberculosis provide an accurate measure of protective efficacy against *M. tuberculosis* infection".

Three questions were addressed:

1) Were there any differences in IFN-γ production in two macaque species: rhesus macaques and cynomolgus macaques?
2) Was IFN-γ production in macaques similar to that observed in human clinical trials?
3) Were IFN-γ measurements (frequency of IFN-γ secreting cells or concentration of IFN-γ secreted) in macaques correlated with protection against *M. tuberculosis* infection?

The findings from these questions are summarised and discussed below.

6.1.1 Differences in IFN-γ responses between two macaque species

During BCG vaccination it was shown that rhesus macaques have higher frequencies of PPD-specific IFN-γ secreting cells and increased concentrations of IFN-γ secreted after PPD-stimulation of diluted whole blood compared to cynomolgus macaques. During *M. tuberculosis* infection, the frequencies of IFN-γ-secreting cells were also higher in rhesus macaques compared to levels detected in cynomolgus macaques. However, similar concentrations of IFN-γ were observed in the PPD-stimulated cell supernatants in both

species of macaques after *M. tuberculosis* challenge. Therefore, the trend was that **IFN-γ responses were higher in rhesus macaques compared to cynomolgus macaques during BCG vaccination and *M. tuberculosis* infection**, especially with respect to the number of antigen-specific cells secreting this cytokine.

The analysis of TB-specific IFN-γ responses in two macaque species has only once been reported in the literature (Langermans *et al.*, 2001). However, their study only assessed IFN-γ production at one time point, 13 weeks after BCG vaccination, showing that rhesus macaques produced more than twice the amount of PPD-specific IFN-γ compared to cynomolgus macaques (Langermans *et al.*, 2001). The challenge experiments reported by Langermans *et al.* also used a large dose of 3,000 cfu *M. tuberculosis* delivered via intratracheal instillation compared to the aerosol delivery of a small number (30-75 cfu) of *M. tuberculosis* bacilli in the studies conducted at the Health Protection Agency (HPA) and reported in this thesis. This is the first time that a simultaneous comparison of IFN-γ responses between two macaque species after aerosol *M. tuberculosis* infection has been reported.

One possible explanation for differences between the macaque species could be that the antibodies used in the IFN-γ ELISPOT and ELISA assays have a higher affinity to rhesus macaque IFN-γ than cynomolgus macaque IFN-γ. The same antibody clones were used for both the ELISPOT and ELISA assays. The combination of monoclonal antibodies GZ-4 for capture and 7-B6-1 for detection used in this thesis have also been shown by other researchers to result in the detection of the highest levels of monkey IFN-γ (Makitalo *et al.*, 2002). However, the IFN-γ molecule is identical in rhesus macaques and cynomolgus macaques (Tatsumi & Sata, 1997; Villinger *et al.*, 1995), discounting the

argument of different antibody affinities contributing to the differences seen between the two macaque species.

6.1.2 Comparison of IFN-γ responses in macaques and humans

To date, there have been no publications directly comparing TB vaccination responses in humans and macaques using the same assays, time-points and vaccine preparations. In the studies reported in this thesis, data from macaques immunised with BCG and MVA85A were compared with results generated in human clinical trials conducted in Oxford.

In a pilot study using two rhesus macaques and two cynomolgus macaques vaccinated with BCG, the rhesus macaques showed frequencies of PPD-specific IFN-γ-secreting cells similar to humans. The cynomolgus macaques, on the other hand, had much lower responses.

Using a larger cohort of 12 rhesus macaques, the frequency of PPD-specific and Ag85A-specific IFN-γ-secreting cells followed the same trends in both macaques and humans after BCG vaccination. After MVA85A-immunisation, the kinetics of the response were similar but the magnitude was lower in rhesus macaques compared to responses in human studies. One week after MVA85A-immunisation, the responses to PPD and Ag85A were not significantly different between the two species, but beyond week 1, the Ag85A responses were statistically higher in humans than rhesus macaques. With Ag85A being the antigen for the MVA85A vaccine, this observation showed a distinct difference between the species.

The finding that rhesus macaques showed different IFN-γ responses to Ag85A peptides post-MVA85A-immunisation to humans is extremely important in the calibration of the model to assess TB vaccines. As the rhesus macaque IFN-γ immune response mimicked the kinetics of human responses, the role of these animals in the testing of novel TB vaccines remains relevant. However, due to the magnitude of IFN-γ responses being lower, care has to be exercised in interpretation of results. Because only one in 10 infected individuals develop TB in their lifetime, large numbers of individuals will be required to demonstrate efficacy of any new vaccine in phase III clinical trials (Sander & McShane, 2007). However, macaques can be challenged in a controlled manner. Therefore, a balance needs to be assessed over risks and benefits in order to maximise the usefulness of non-human primate studies.

6.1.3 IFN-γ as a correlate of protection

6.1.3.1 IFN-γ responses during vaccination

During BCG vaccination, the animals with the lowest peak frequencies of PPD-specific IFN-γ secreting cells were also statistically significantly likely to survive longer post-*M. tuberculosis* challenge. This indicates an inverse relationship between the number of cells secreting IFN-γ and survival. However, this observation was made using only 6 animals, so should be followed up using a larger cohort of animals.

After MVA85A boosting of BCG primed animals, the rhesus macaque (animal K65) that had the most PPD- and Ag85A-specific IFN-γ secreting cells and the highest concentration of PPD-specific IFN-γ secreted in 6 day diluted whole blood supernatant survived for a year post-*M. tuberculosis* challenge, there was no evidence of a trend of increased IFN-γ responses in animals that survived for periods beyond 16 weeks post-

challenge than those which met humane clinical endpoints before 16 weeks. Therefore, it was concluded that the frequency of Ag85A- or PPD-specific IFN-γ-secreting cells or the concentration of IFN-γ secreted in 6-day PPD-stimulated diluted whole blood induced after MVA85A vaccination did not correlate with protection.

There is certainly a minimum requirement for IFN-γ to protect against *M. tuberculosis* disease (Rook *et al.*, 2006), which can be enhanced by booster vaccines that increase IFN-γ levels (Goonetilleke *et al.*, 2003; Tanghe *et al.*, 2001). However, other studies show that a plateau of protective effect is rapidly reached, where further enhancement of IFN-γ production is not helpful (Leal *et al.*, 2001; Skinner *et al.*, 2003). Therefore, the plateau of response may possibly only have been breached by one animal, K65, resulting in enhanced survival. However, further studies would be needed to confirm this hypothesis.

Others have found that increasing the IFN-γ response does not necessarily increase survival (Fonseca *et al.*, 2007; Majlessi *et al.*, 2006; Pardini *et al.*, 2006; Young *et al.*, 2002). However, as seen with the MVA85A vector in the rhesus macaque studies, these observations suggest that IFN-γ on its own does not correlate with protection in these models, meaning that further work is needed to determine what represents a correlate of protection against TB disease.

6.1.3.2 IFN-γ responses after *M. tuberculosis* challenge of vaccinated animals

Despite the early PPD-specific IFN-γ responses after *M. tuberculosis* challenge in the BCG/MVA85A-vaccinated animals in the present study, compared to BCG-only or unvaccinated control animals, this vaccination approach did not afford significant protection against *M. tuberculosis*. The ability of *M. tuberculosis* to limit activation of

macrophages by IFN-γ suggests that the amount of IFN-γ produced by T cells may be less predictive of outcome than the ability of the cells to respond to this cytokine (Flynn & Chan, 2001a). The presence of a functional IFN-γ receptor is essential for the recovery of mice from BCG infection (Kamijo *et al.*, 1993).

6.1.4 Conclusion

This thesis has documented IFN-γ responses following *M. tuberculosis* infection and after vaccination with BCG and the novel 'booster' vaccine, MVA85A. The results have shown that IFN-γ responses in rhesus macaques during vaccination are similar in kinetics to those observed in humans. However, the magnitude of IFN-γ secreting cells post-MVA85A boost were lower in rhesus macaques compared to human responses. Work has also proved that IFN-γ is an important marker showing that immunity has been generated. However, neither the frequency of IFN-γ-secreting cells nor the concentration of IFN-γ secreted in a vaccination regime was indicative of protection. As the data reported in this thesis discount the hypothesis that IFN-γ alone is a correlate of protection against *M. tuberculosis* infection, this leaves the elusive immune mechanism still to be discovered. However, it remains possible that IFN-γ is just one component of many that may make a protective biosignature, as proposed by others (Beveridge *et al.*, 2008).

6.2 IMPROVEMENTS AND LIMITATIONS

A main limitation to these studies is the measurement of IFN-γ responses in peripheral blood, as the disease site in TB is mainly in the lungs. Although peripheral T cells and monocytes can be influenced by pulmonary pathology, as they travel through the lung many times each day, the evaluation of their function may provide rather indirect information on what happens at the site of disease (Vanham *et al.*, 1997). Using BCG-MVA85A vaccination in mice, Ag85A-specific T cell responses in the lung lymph nodes

(LLN) were found to correlate better with protection in the lung than splenic immune responses (Goonetilleke *et al.*, 2003). This observation has also been reported using Ag85A contained in an adenovirus vector where responses in the lung correlated better with survival than splenic responses (Forbes *et al.*, 2008). These data suggest that the frequency of antigen-specific IFN-γ-secreting T cells in the vicinity of the lung may constitute a better indicator of protection in lung than systemic responses against pulmonary TB. It may be that the lymphocytes resident in the LLN can effectively control *M. tuberculosis* infection limiting its spread systemically.

The readouts from the IFN-γ assays used in this thesis were the frequencies of antigen-specific IFN-γ cells and the concentration of IFN-γ secreted. Although the levels of the cytokine secreted may be important, receptors for the cytokine are also required. Ratios of cytokine:cytokine receptor may be more predictive of disease outcome then the measurement of the cytokine alone (McDermott, 2001). Other investigators have measured IFN-γ-induced responses, which assess the integrity of the IFN-γR signalling pathway using monokine induced by gamma interferon (MIF) (Abramo *et al.*, 2006). This method would, therefore, test the functioning of IFN-γ, instead of purely its production, and thus may be a more suitable measure of IFN-γ function.

Looking at the actual cytokine network *in vivo* may also prove beneficial in assessing responses during *M. tuberculosis* infection (Verbon *et al.*, 1999). After prolonged stimulation, such as with a chronic infection, elevated cytokine levels may be evident without additional stimulus (Morosini *et al.*, 2003). As such, circulating levels of IFN-γ in the plasma have been shown to correlate with disease severity (Sahiratmadja *et al.*, 2007a). These elevated levels may be generated following prolonged stimulation, as would be expected in *M. tuberculosis* infection.

Work described in this thesis was routinely carried out using unfractionated PBMC or whole blood. Therefore, the T-cell subsets involved in immunity were not routinely assessed. A vaccine-induced $CD8^+$ T cell response may provide an important role in protective immunity (Behar *et al.*, 2007). Another important cell subset is CD1-restricted T cells. Lipid antigens which are abundantly expressed by mycobacteria are highly specific for CD1 molecule activation (Behar & Porcelli, 2007). The primate model of *M. tuberculosis* infection may prove an excellent approach for studying these responses as rodent models lack group 1 CD1-restricted T cells that function primarily in the adaptive immune response (Behar & Porcelli, 2007). Assays to monitor the contribution of individual cell types in the production of IFN-γ may therefore identify differences in vaccination approaches and establish the ideal repertoire of cells required for protection against *M. tuberculosis* infection.

In this thesis, the focus was to address solely IFN-γ responses. However, the requirement of a balance of Th1 and Th2 cytokines, governed by IFN-γ, IL-10 and IL-4 to prevent tissue destruction, has been suggested by a model used to predict cell-mediated immune regulatory mechanisms during human TB (Wigginton & Kirschner, 2001). Although the measurements of IL-1β, IL-2, IL-6, IL-10, IL-12 and TNF-α were undertaken in the *M. tuberculosis* challenged macaques, levels were only determined over a 12 week time-course of animals that did not succumb to rapid disease progression. Looking at a larger range of cytokines may yield more answers about the immune response, and why some vaccines fail to protect against *M. tuberculosis* challenge. Implementing multiplex analysis, such as using luminex assays, during the vaccination stage would assist in further defining vaccine-induced immune responses.

6.3 FUTURE WORK

6.3.1 Cytokines beyond IFN-γ

6.3.1.1 Th1 cytokines

Although IFN-γ is a major cytokine necessary for the control of *M. tuberculosis* infection, obviously there are others involved. IL-12 is critical in the development of a Th1 response and the production of IFN-γ, but additionally TNF can synergise with IFN-γ to activate macrophages (Collins & Kaufmann, 2001). Therefore, future studies that assess cytokines that induce or work alongside IFN-γ may yield further evidence on potential correlates of protection.

Measuring another cytokine such as IL-2 may prove beneficial in measuring the quality of IFN-γ-secreting cells instead of the quantity. In studies of human responses following vaccination with tetanus or hepatitis B virus two types of Th1 cells were examined; those that produce IFN-γ and IL-2, and those that produced only IL-2 (De Rosa *et al.*, 2004). The IFN-γ- and IL-2-producing Th1 cells were found to be short-lived *in vitro* and *in vivo*. In contrast, the Th1 cells that only produce IL-2 efficiently developed into long-term memory cells (De Rosa *et al.*, 2004). Using polyfunctional T cell analysis by flow cytometry, in human studies the Ag85A-specific IFN-γ-secreting cells in BCG/MVA85A vaccinated volunteers were also shown to secrete IL-2/TNF-α/MIP-1β (Beveridge *et al.*, 2007) and IL-2/TNF-α/IL-17 (Hawkridge *et al.*, 2008). Therefore, it is possible that the IFN-γ-secreting cells detected in the rhesus macaques following MVA85A-boosting were also secreting these cytokines and so were contributing to the immune response by more than solely the secretion of IFN-γ.

6.3.1.2 Th2 cytokines

Th2 cytokines have been detected in TB patients during the chronic stage of disease. The late appearance of Th2 cells does not necessarily imply a pathogenic role, but may suggest that they participate in down-regulation of the protective Th1 response to avoid exaggerated pathologic consequences (Kaufmann & Andersen, 1998). Blood monocytes and lung granuloma macrophages from patients with active tuberculosis express TGF-β, a Th2 cytokine, and excessive activity of this cytokine may underlie depressed T cell responses (Toossi *et al.*, 1995). Mean titres of Th1 cytokines (IFN-γ and IL-2) in mild and moderate TB patients appeared higher than in those with advanced disease, whereas moderate and advanced patients showed the higher levels of a Th2 cytokine (IL-4) in comparison to mild cases (Dlugovitzky *et al.*, 1997). A Th2 immune component of a TB vaccine may therefore prove advantageous in preventing potential immunopathology caused by an excess of pro-inflammatory cytokines.

6.3.1.3 Th17 cells

A new class of T-helper cell that complements the Th1 and Th2 subsets has been proposed, named Th17 due to its secretion of IL-17. The production of IL-17 during *M. tuberculosis* infection is primarily by γδ T cells after stimulation with IL-23 (Lockhart *et al.*, 2006). Evidence suggests that IL-17 is central to lymphocyte migration to the lung (Kolls & Linden, 2004), including after BCG vaccination (Khader *et al.*, 2007). Therefore, directing T lymphocytes to the lung by activating the IL-17-producing Th17 cells could improve the protective potential of novel vaccine candidates (Kaufmann, 2006b). It has been suggested that IL-17 is not involved in protection against *M. tuberculosis*, but instead regulates inflammation (Khader & Cooper, 2008). Due to IFN-γ affecting the production of IL-17 (Cruz *et al.*, 2006), levels of these two cytokines may be critical to the inflammatory outcome of any mycobacterial infection (Khader &

Cooper, 2008). Increased levels of IL-17 were found when boosting BCG vaccination responses with MVA85A in mice, but no protection was observed against a virulent *M. tuberculosis* infection (Romano *et al.*, 2006). However, challenge was performed intravenously, so the cells in the lung were not preferentially targeted during infection. In the future investigating the effect of IL-17 on immune responses in protected vs. non-protected macaques, especially after aerosol *M. tuberculosis* challenge, may be useful.

6.3.2 Involvement of regulatory T-cells

TB disease may be accompanied by (a) the wrong type of regulatory T cell activity, compromising essential effector mechanisms, or (b) insufficient regulatory activity, which therefore results in pulmonary immunopathology (Doherty & Rook, 2006).

In a study to determine whether regulatory T cells affected the immune response induced by BCG vaccination, cells were inactivated using anti-CD25 antibody (Quinn *et al.*, 2008). Although this resulted in increased numbers of IL-2 producing $CD4^+$ cells after BCG vaccination and an accelerated response after *M. tuberculosis* challenge, the level of protection remained unaltered compared to control mice (Quinn *et al.*, 2008). These results suggest that regulatory T cells do not affect the efficacy of BCG vaccination.

When BCG responses in humans were boosted with MVA85A, the immunoregulatory cytokine transforming growth factor beta 1 (TGF-β1) was downregulated (Fletcher *et al.*, 2008). This may allow for the induction of increased effector responses, such as those elevated frequencies of IFN-γ-secreted cells observed in the rhesus macaques in this thesis; especially as the effect was observed 7 days post-MVA85A boost.

In infection the role of regulatory T cells may be more apparent as *M. tuberculosis* may exploit the inflammatory process to cause immunopathology and cavitation that

characterise the disease and facilitate further transmission by coughing (Doherty & Rook, 2006). If this is the case, then perhaps in the lungs at least, there is too little regulatory T-cell activity (Doherty & Rook, 2006). In TB patients, regulatory T cells have been successfully isolated from PBMC at higher levels compared to those from healthy individuals with latent TB or uninfected controls (Hougardy *et al.*, 2007). It has been shown in mice that, after aerosol *M. tuberculosis* infection, regulatory T cells accumulated in the lung and pulmonary lymph nodes and suppressed the immune response that controls *M. tuberculosis* growth, thus having a negative effect on the control of TB disease (Scott-Browne *et al.*, 2007). Therefore, a balance is required where regulatory T cells are required to prevent destructive inflammatory processes, but at the same time not suppress protective immune responses.

6.3.3 Other antigens

Future work could include looking at other antigenic components of *M. tuberculosis* in order to stimulate a broader range of immune responses. In the macaques, only responses to PPD and selected peptides were measured. Whereas PPD predominantly induces a $CD4^+$ αβ T-cell response, there is little or no increase in γδ T cells (Fenton & Vermeulen, 1996; Tsukaguchi *et al.*, 1995). However, BCG also contains lipid antigens, which can induce immune responses (Watanabe *et al.*, 2006). Vaccination with BCG has been shown to induce γδ T-cells in cynomolgus macaques (Cairo *et al.*, 2007), rhesus macaques (Shen *et al.*, 2002), pigs (Lee *et al.*, 2004) and humans (Hoft *et al.*, 1998). γδ T cells can constitute 5% of T cells in lymphoid organs and 1-5% of circulating blood lymphocytes; they are also preferentially expanded by live mycobacteria suggesting they may play a role in the primary immune response to infection (Orme *et al.*, 1993a). Additionally, γδ T cells secrete IFN-γ before αβ T cells, so may contribute to early control of infection by production of this cytokine (Vanham *et al.*, 1997). γδ T cells have

also been shown to increase after *M. tuberculosis* infection of macaques (Shen *et al.*, 2002).

6.3.4 Antibody induction

Anti-vector antibodies were measured in this thesis, showing an interesting observation that the MVA85A-boosted animal with the lowest anti-vector antibodies (K65) was also the only animal that survived for a year post-challenge (appendix 21, section 8.21). This animal also had the highest IFN-γ responses. Due to being only a single animal, it is difficult to ascertain the significance of this finding. However, future work may look into antibody responses of the insert protein to determine whether responses are biased towards the vector or the TB protein, and to analyse the importance of the overall humoral response with respect to survival. When MVA vectors containing cytomegalovirus proteins have been assessed in rhesus macaques, both antibody and cellular responses were observed concomitantly (Yue *et al.*, 2008); thus, it is possible that this observation will also be seen in MVA85A-boosted macaques.

6.3.5 Mycobacteria-killing assays

Antigen-specific IFN-γ T cell responses are useful for confirming the induction of the pattern of cellular immunity associated with protection. These will be employed in the initial phase I/II human studies of vaccines, but it has been shown in studies conducted in this thesis and by others (Britton & Palendira, 2003) that increased IFN-γ production and protection do not always correlate in experimental models of TB. IFN-γ alone is not sufficient to activate human infected macrophages to kill *M. tuberculosis*, and a more global *in vitro* mycobacterial killing assay may measure the effect of the multiple factors needed to control the infection (Britton & Palendira, 2003). IFN-γ production has been

shown not to correlate with mycobacterial inhibition (Hoft *et al.*, 2002), highlighting that this cytokine alone is not solely responsible for killing or stasis of mycobacteria.

6.3.6 Material available for the future assessment of immune parameters

The storage of material (PBMC, tissue lymphocytes, sera, saliva and stimulated blood supernatants) generated during this project will permit some of the suggested analyses to be undertaken. Where samples are not available, then future studies undertaken in non-human primates may make provisions to ensure that the appropriate samples are available. One such example would be to assess immune responses in the lungs, the primary site of infection. In studies documented in this thesis, lungs were fixed in formaldehyde for magnetic resonance imaging (MRI) scanning and histology, or used to assess bacterial load. Therefore, future studies will need to address the benefits of immunological analysis in addition to work carried by other disciplines where samples are limited to ensure that the maximum information continues to be gained from the macaques.

Of utmost important is the fact that material is stored from animals that have been shown to be protective, and from those that were not. This will allow future studies to discriminate between the two groups, and therefore should greatly aid interpretation of results in the hunt for a correlate of protection.

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8 APPENDICES

8.1 APPENDIX 1: Challenge doses

The following tables show the doses of *M. tuberculosis* delivered to the macaques estimated from the circulating concentration of bacilli and the inhaled volume of the animals.

Challenge studies:

	Estimated dose (cfu retained)							
	1st challenge study		2nd challenge study		3rd challenge study		4th challenge study	
Rhesus macaques	D53	500	D19	70	D12	75		
	D80	30	C54	40	D42	45		
	D31	30	D60	40	D28	45		
Cynomolgus macaques	9909187	30	109181	40	1163	40	0111231	75
	9909013	30	109027	40	1111	40	0201011	75
	4313	30	109203	40	2151	40	0201191	75

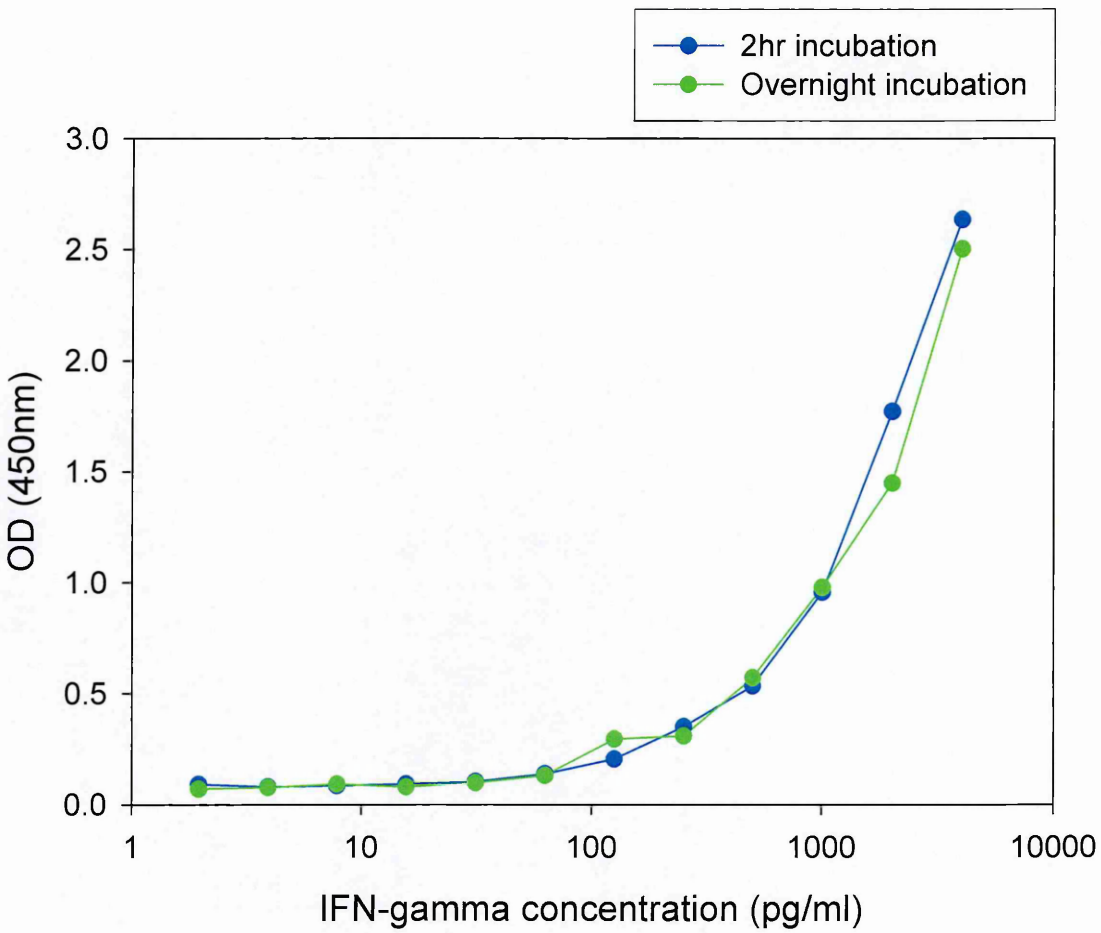
Vaccine efficacy study:

		Estimated dose
K44	K50	50 cfu
K20	K61	
K79	K59	
K43	K69	
K32	K54	
K47	K62	
K52	K86	
K65	K80	

Acknowledgements:

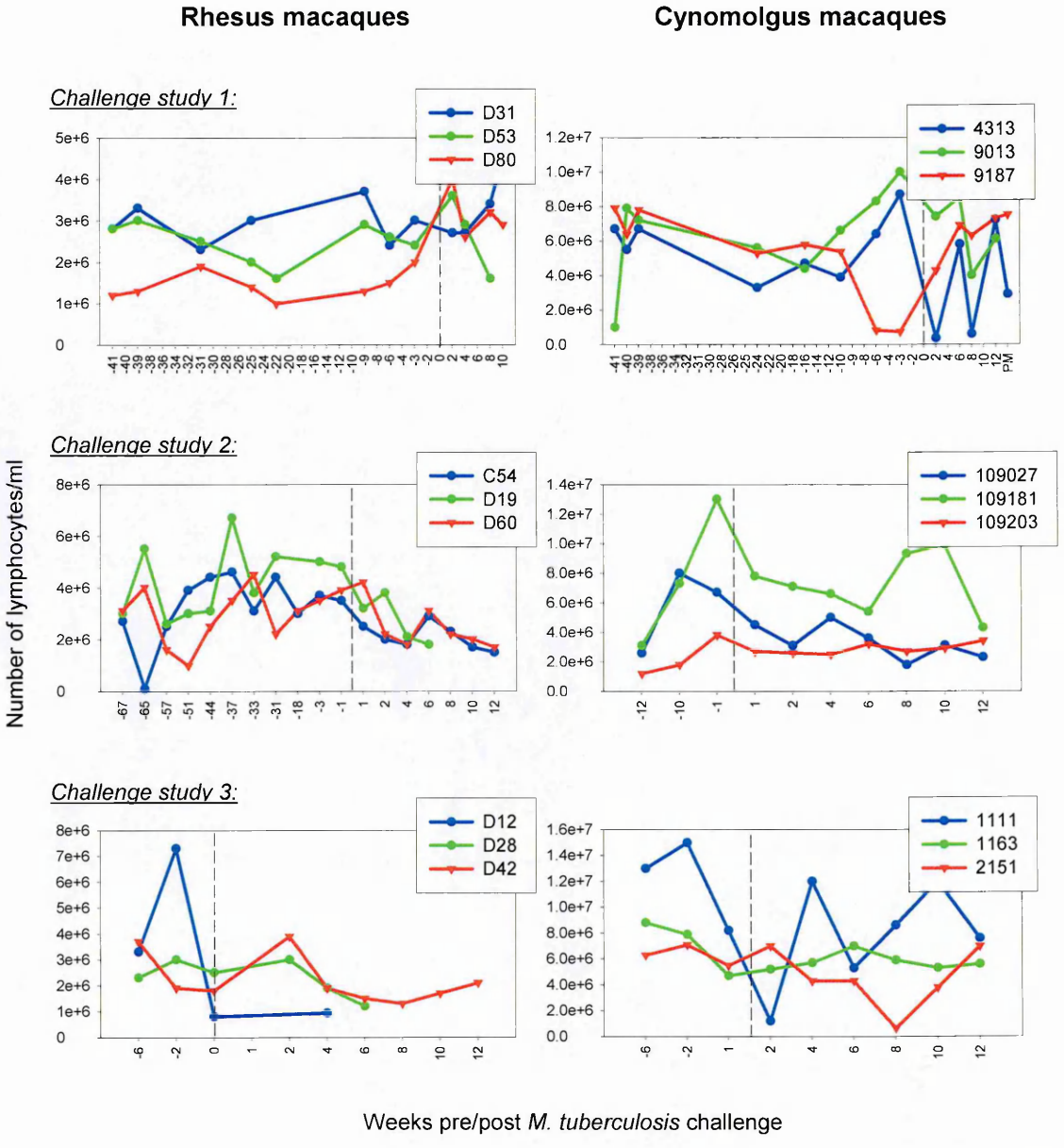
This data was supplied by Ann Rawkins, Simon Clark and Graham Hatch.

8.2 APPENDIX 2: Effect of ELISA incubation conditions



Effect on incubating samples for 2 hours at room temperature or overnight at 4°C on absorbance readings of a standard curve of known IFN-γ concentrations. No difference between the two incubations were found, so all subsequent analysis was carried out overnight at 4°C.

8.3 APPENDIX 3: Lymphocyte counts during *M. tuberculosis* infection

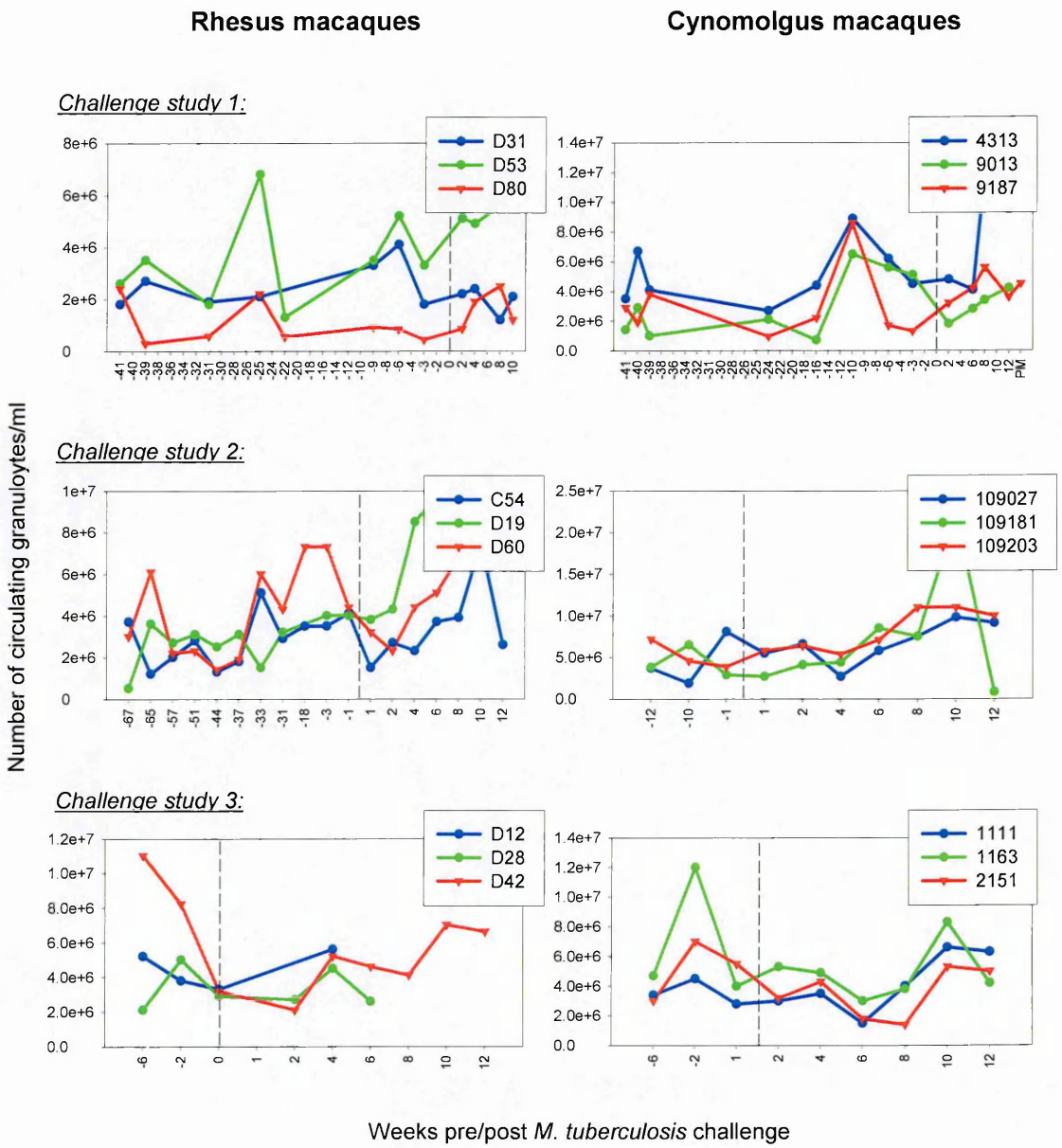


Lymphocyte counts (assessed by forward scatter/side scatter) on the flow cytometer during *M. tuberculosis* infection.

Acknowledgements:

Immunophenotyping results were supplied by Karen Gooch.

8.4 APPENDIX 4: Granulocyte counts during *M. tuberculosis* infection

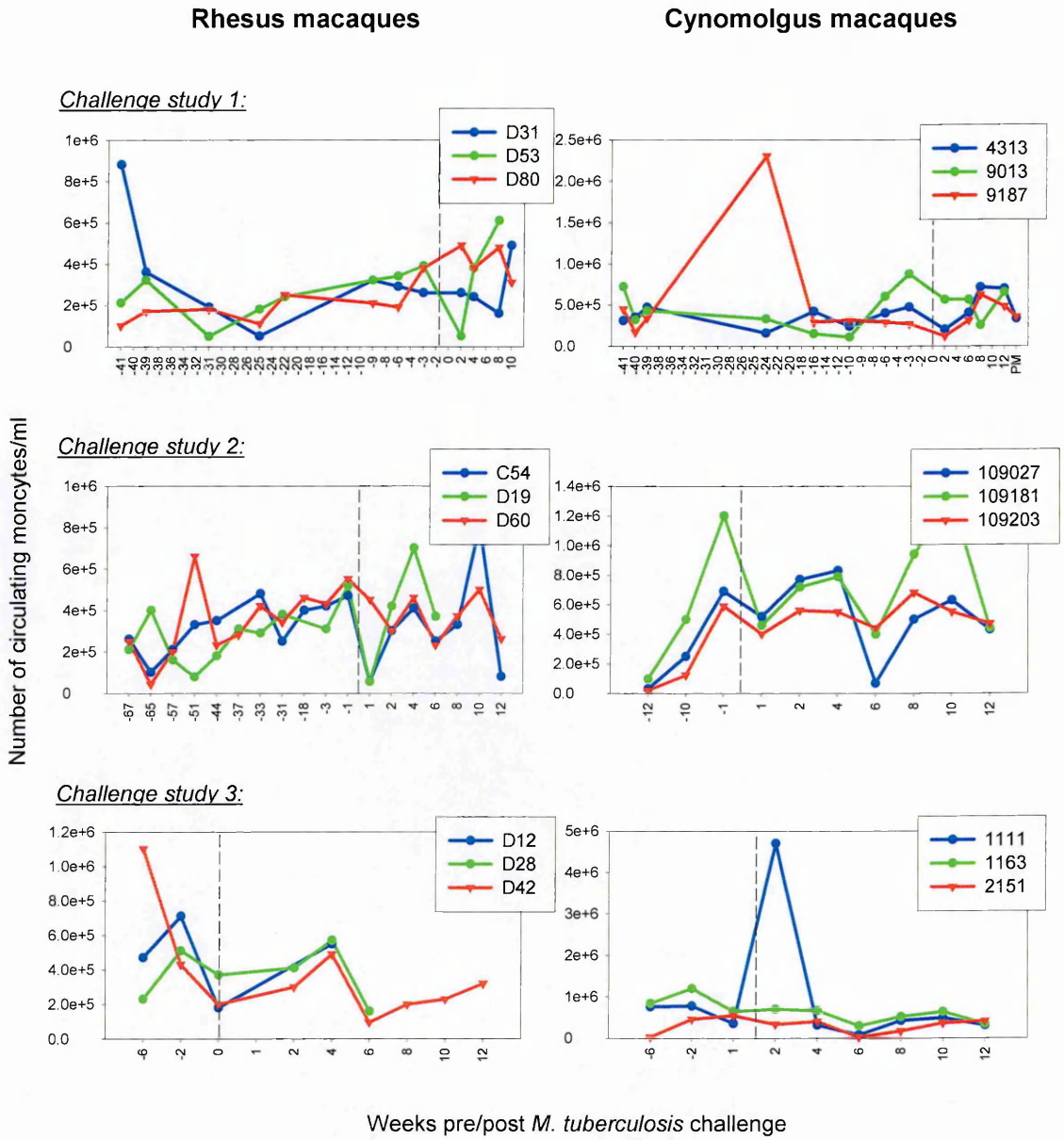


Granulocyte counts (assessed by forward scatter/side scatter) on the flow cytometer during *M. tuberculosis* infection.

Acknowledgements:

Immunophenotyping results were supplied by Karen Gooch.

8.5 APPENDIX 5: Monocyte counts during *M. tuberculosis* infection

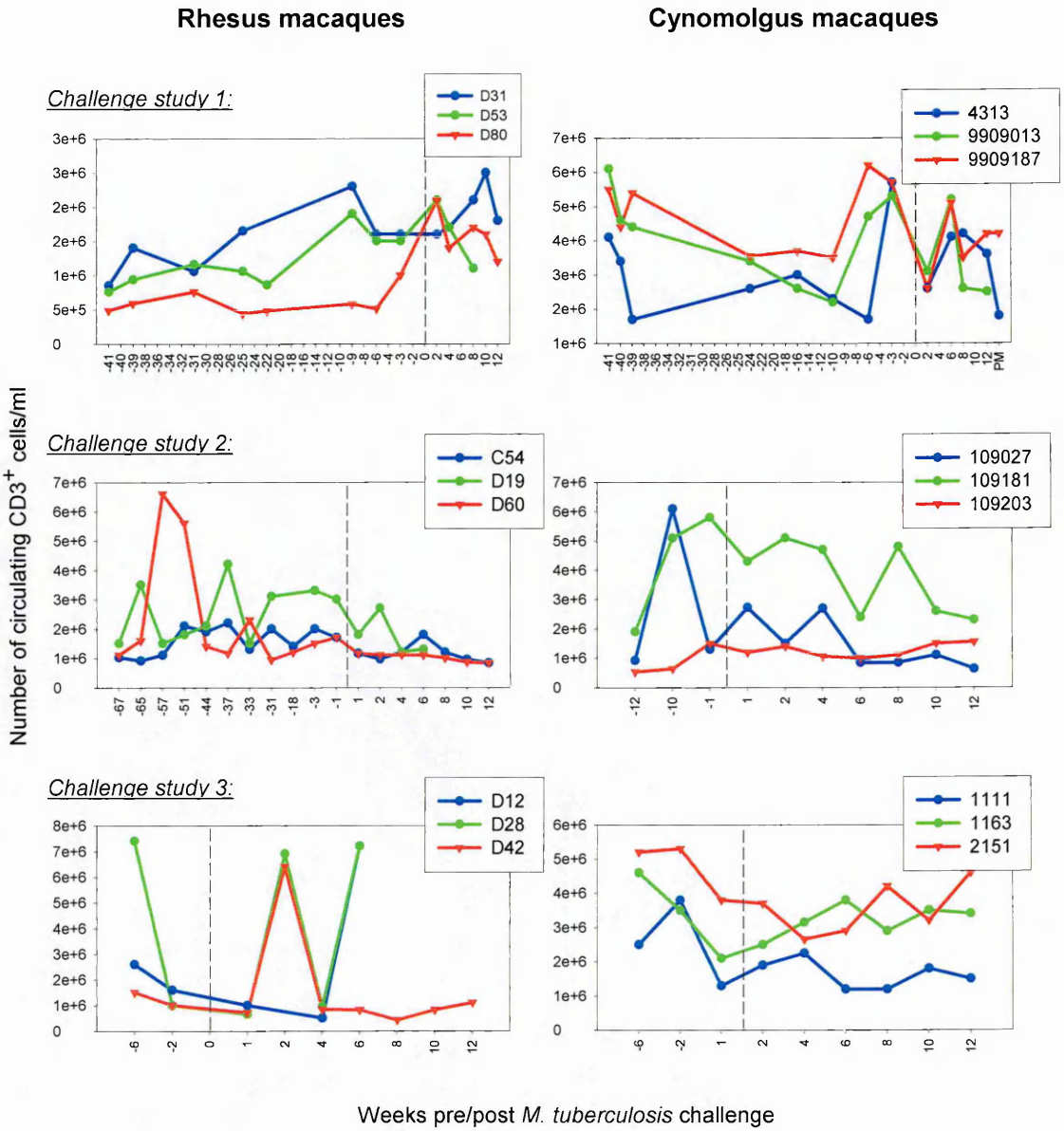


Monocyte counts (assessed by forward scatter/side scatter) on the flow cytometer during *M. tuberculosis* infection.

Acknowledgements:

Immunophenotyping results were supplied by Karen Gooch.

8.6 APPENDIX 6: $CD3^+$ counts during *M. tuberculosis* infection

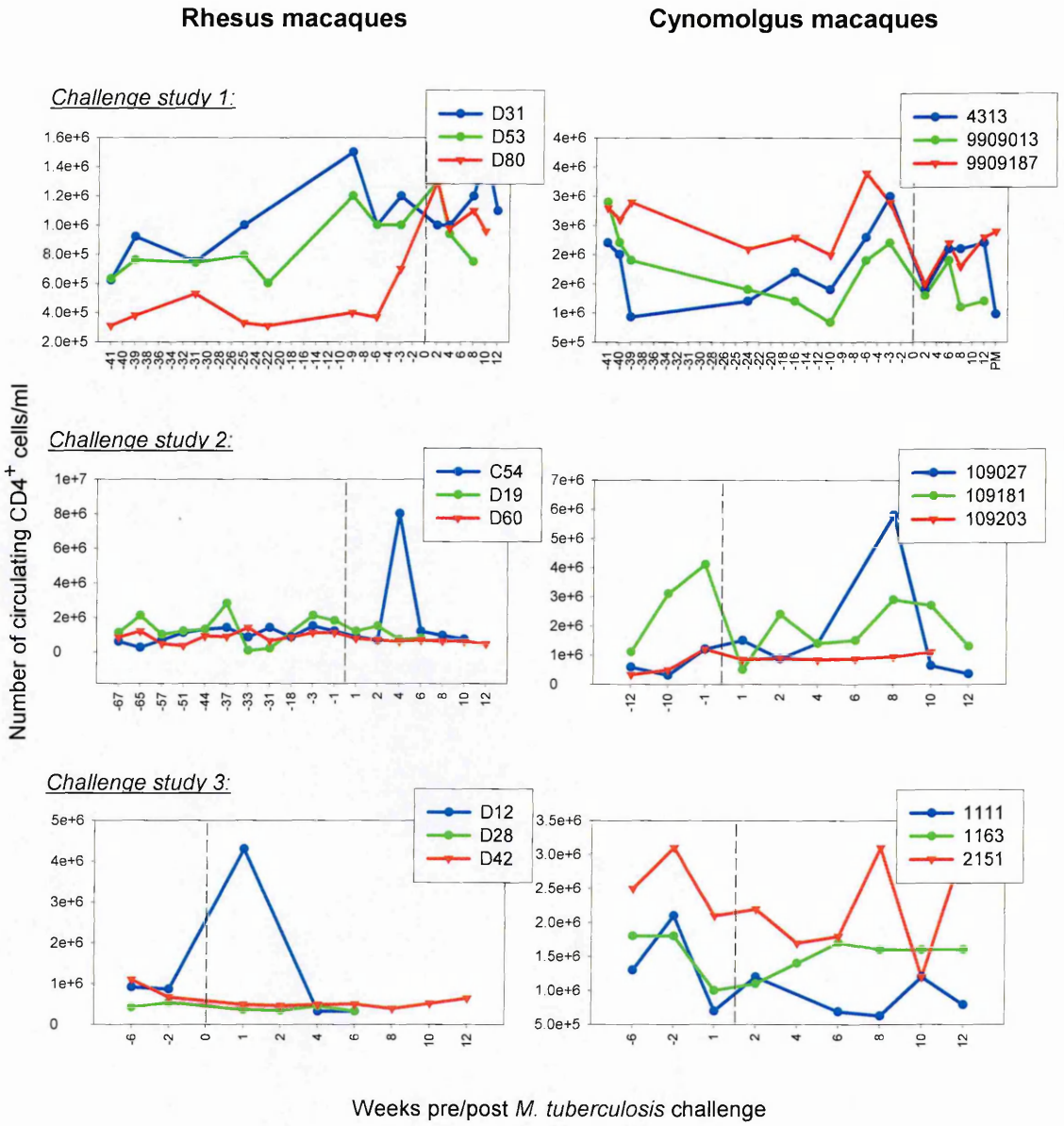


$CD3^+$ counts (assessed by CD3 antibody staining) on the flow cytometer during *M. tuberculosis* infection.

Acknowledgements:

Immunophenotyping results were supplied by Karen Gooch.

8.7 APPENDIX 7: CD4$^+$ counts during *M. tuberculosis* infection

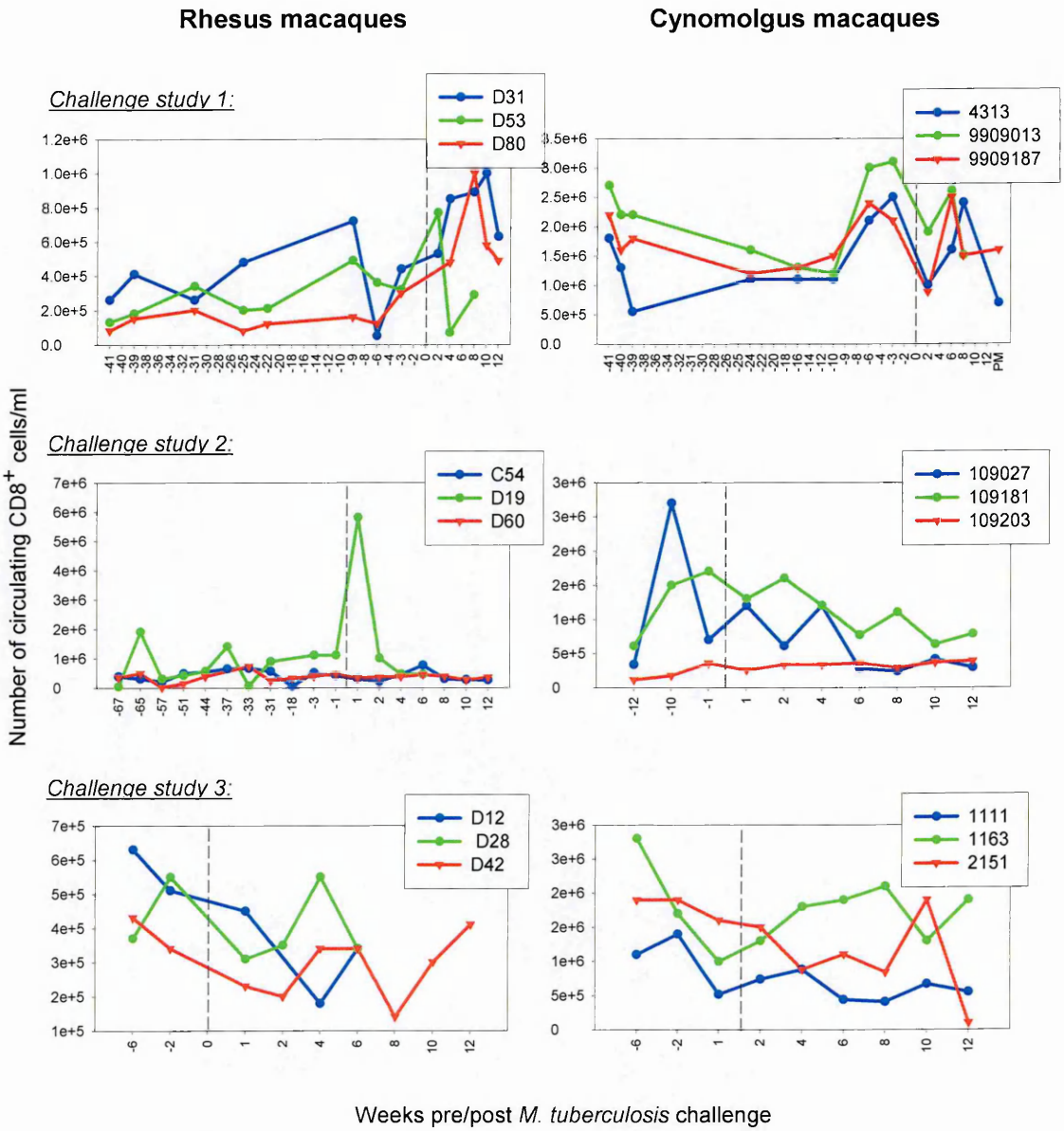


CD4$^+$ counts (assessed by CD4 antibody staining) on the flow cytometer during *M. tuberculosis* infection.

Acknowledgements:

Immunophenotyping results were supplied by Karen Gooch.

8.8 APPENDIX 8: CD8+ counts during *M. tuberculosis* infection

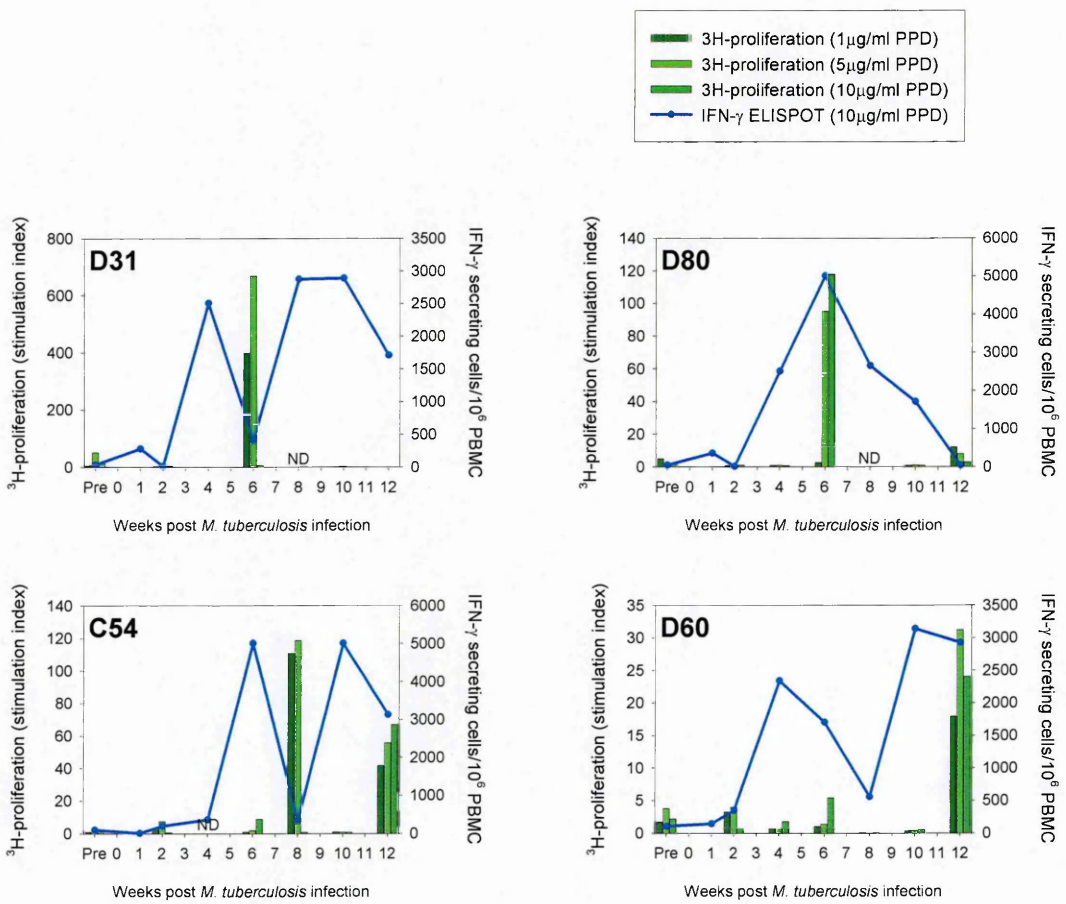


$CD8^+$ counts (assessed by CD8 antibody staining) on the flow cytometer during *M. tuberculosis* infection.

Acknowledgements:

Immunophenotyping results were supplied by Karen Gooch.

8.9 APPENDIX 9: Proliferative responses during *M. tuberculosis* infection (rhesus macaques)



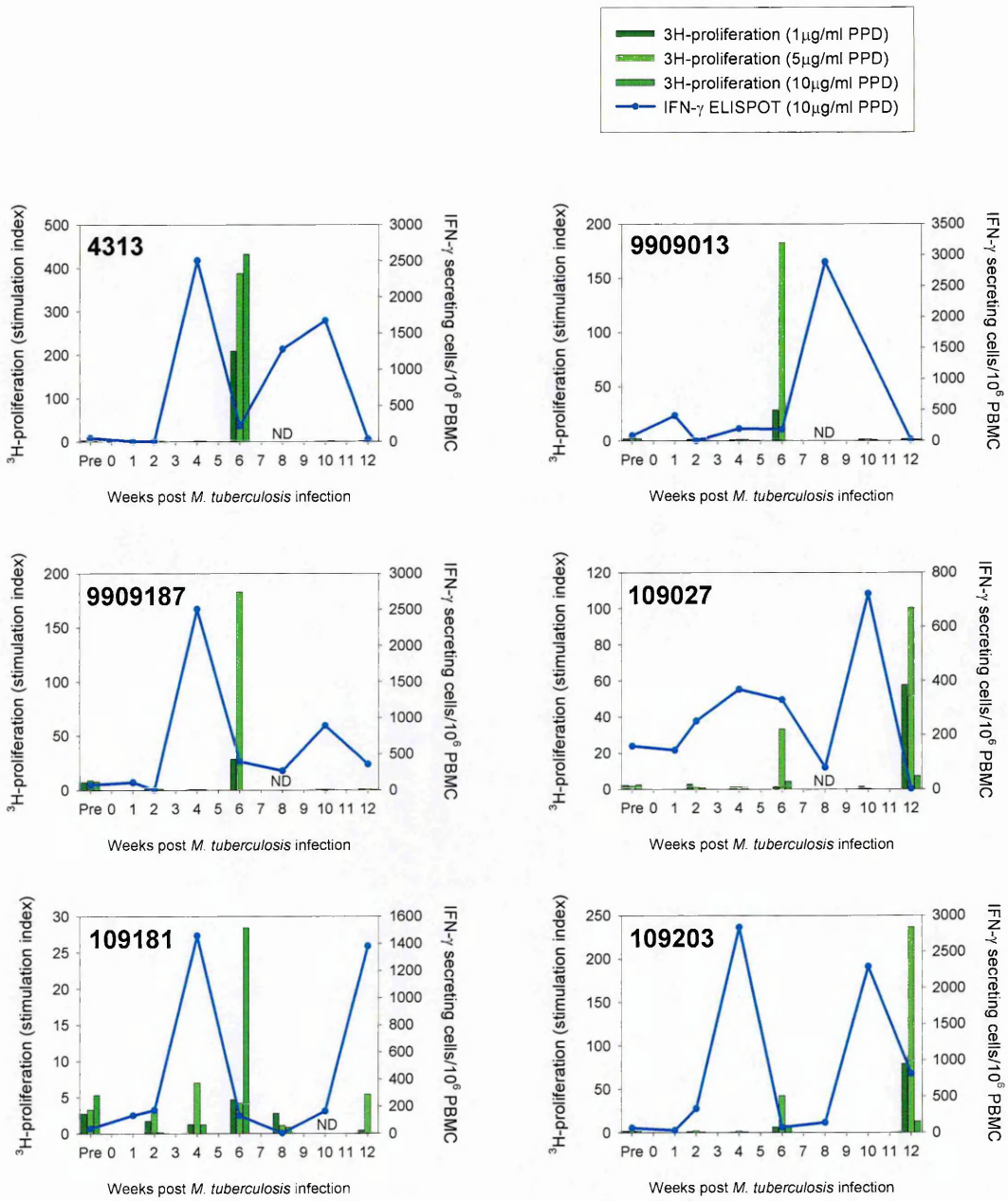
Proliferative responses (as assessed by ^{3}H-incorporation) in rhesus macaques during *M. tuberculosis* infection.

(Note: only animals where a complete timecourse was available are shown).

Acknowledgements:

Proliferation results were supplied by Karen Gooch.

8.10 APPENDIX 10: Proliferative responses during *M. tuberculosis* infection (cynomolgus macaques)



Proliferative responses (as assessed by ^{3}H-incorporation) in cynomolgus macaques during *M. tuberculosis* infection.

(Note: only animals where a complete timecourse was available are shown).

Acknowledgements:

Proliferation results were supplied by Karen Gooch.

8.11 APPENDIX 11: Bacteriological findings in spleen of *M. tuberculosis* infected macaques

Rhesus macaques	
Animal no.	Log_{10} cfu/g
D12	2.53
D28	1.56
D42	1.26
D19	2.79
D60	0
C54	1.35
D53	4.50
D31	2.40
D80	0

Cynomolgus macaques	
Animal no.	Log_{10} cfu/g
1111	0.68
1163	0
2151	0
109027	0
109181	1.70
109203	0
9909187	0
9909013	0
4313	0

Bacteriological loads in spleens of *M. tuberculosis* infected macaques.

Acknowledgements:

Bacteriology results were supplied by Simon Clark.

8.12 APPENDIX 12: Bacteriological findings in hilar lymph nodes of *M. tuberculosis* infected macaques

Rhesus macaques	
Animal no.	Log_{10} cfu/g
D12	4.84
D28	4.99
D42	4.17
D19	4.74
D60	0
C54	3.73
D53	5.51
D31	0
D80	4.12

Cynomolgus macaques	
Animal no.	Log_{10} cfu/g
1111	3.65
1163	3.90
2151	4.15
109027	3.89
109181	Not done
109203	3.56
9909187	4.18
9909013	4.86
4313	3.88

Bacteriological loads in hilar lymph nodes of *M. tuberculosis* infected macaques.

Acknowledgements:

Bacteriology results were supplied by Simon Clark.

8.13 APPENDIX 13: X-ray scores during *M. tuberculosis* infection of macaques

Rhesus macaques									
Week no.	**D31**	**D53**	**D80**	**D19**	**D60**	**C54**	**D12**	**D28**	**D42**
0	0	0	0	0	0	0	0	0	0
2	0	1	0	2	0	0	-	-	-
4	0	3	0	4	0	3	4	3	3
6	0	4	0	4	-	-	4+	4	3
8	0		0		1	3			4
10	0		0		1	3			4
12	0		0		3	3			4+

Cynomolgus macaques									
Week no.	**4313**	**9013**	**9187**	**9203**	**9181**	**9027**	**1111**	**1163**	**2151**
0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	-	-	-
4	0	0	0	0	3	0	0	0	0
6	0	1	1	-	-	-	0	0	0
8	0	1	1	0	3	3	0	0	0
10	0	1	1	0	4	3	2	0	0
12	0	1	1	1	4	3	3	0	0

0 = - no infiltrate
1 = + modest infiltrate on one side only
2 = ++ extensive infiltrate on one side only
3 = +++ modest infiltrate on both sides of lungs
4 = ++++ extensive infiltrate on both sides of lungs
4+ will be used to show if the condition seen previously has worsened

X-ray scores of macaques during *M. tuberculosis* infection

Acknowledgements:

X-rays results were analysed by Fergus Gleeson (Churchill Hospital, Oxford).

8.14 APPENDIX 14: Bacteriological counts in BCG-vaccinated macaques

Rhesus macaques:

	Bacteriological count (cfu/g or cfu/ml)	
	Animal 1040	**Animal 1252**
Spleen	0	0
Liver	0	0
Kidney	0	0
Mesenteric LN	0	0
Hilar LN	0	0
Left axillary LN	0	0
Right axillary LN	0	0
Saliva	0	0
Urine	0	0
Faeces	0	0

Cynomolgus macaques:

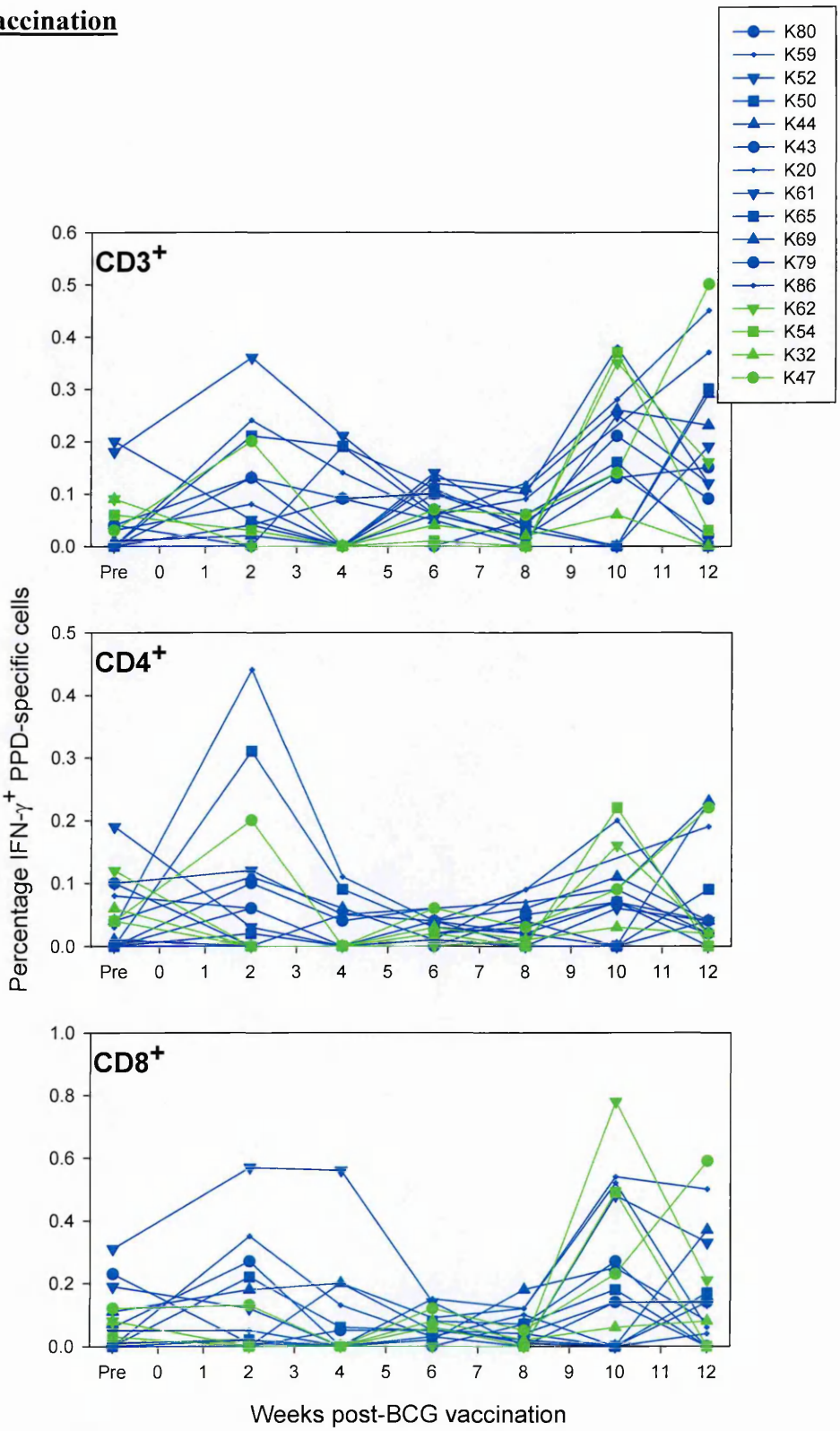
	Bacteriological count (cfu/g or cfu/ml)	
	Animal 1007	**Animal 9911021**
Spleen	0	0
Liver	0	0
Kidney	0	0
Mesenteric LN	0	0
Hilar LN	0	0
Left axillary LN	0	0
Right axillary LN	0	0
Saliva	0	0
Urine	0	0
Faeces	0	0

Bacteriological counts in two rhesus macaques (1040 and 1252) and two cynomolgus macaques (1007 and 9911021) which had been vaccinated with BCG for 74 weeks.

Acknowledgements:

Bacteriology results were supplied by Simon Clark.

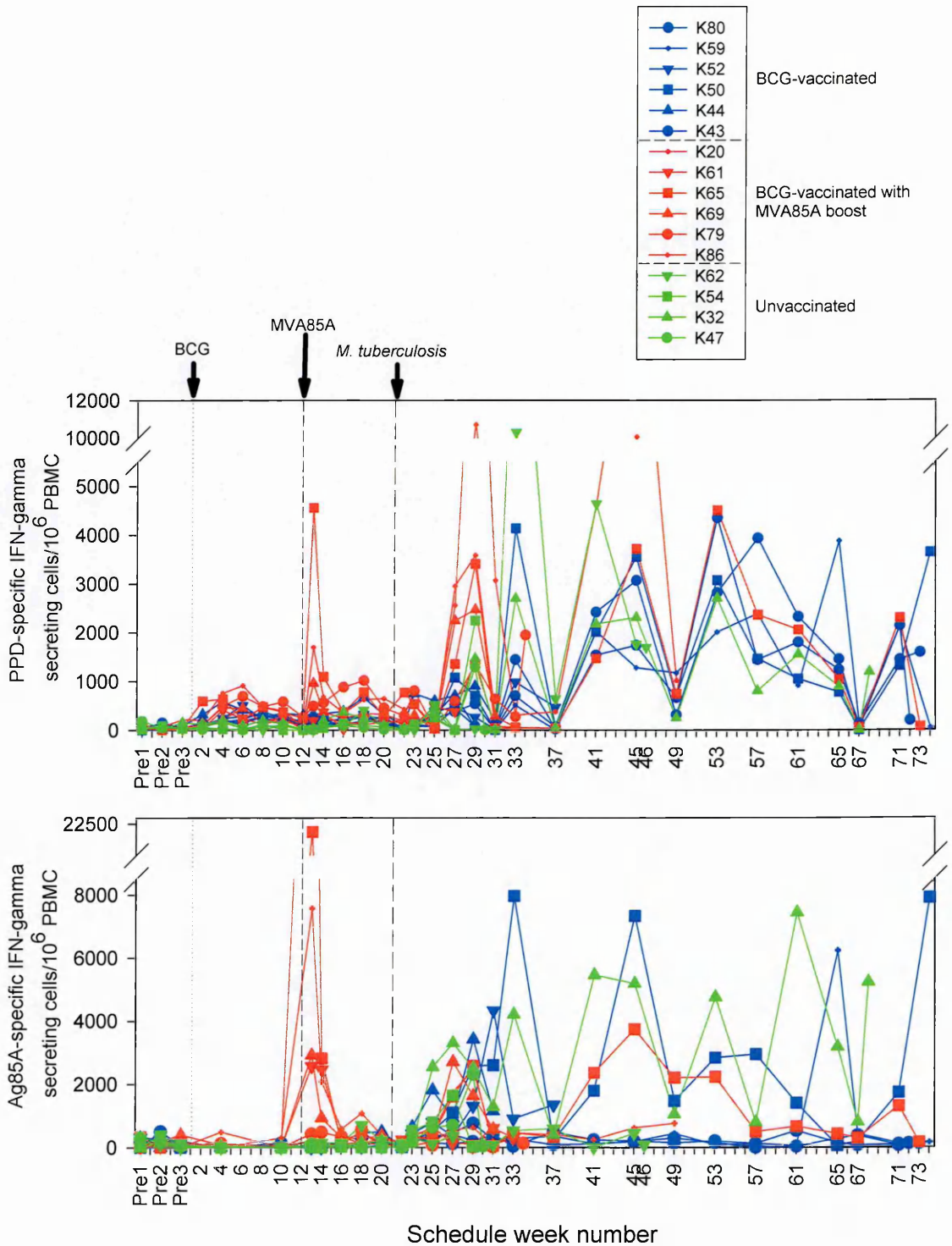
8.15 APPENDIX 15: Intracellular cytokine staining results during BCG vaccination



Intracellular cytokine staining results of rhesus macaques that were vaccinated with BCG (blue lines) or unvaccinated (green lines).

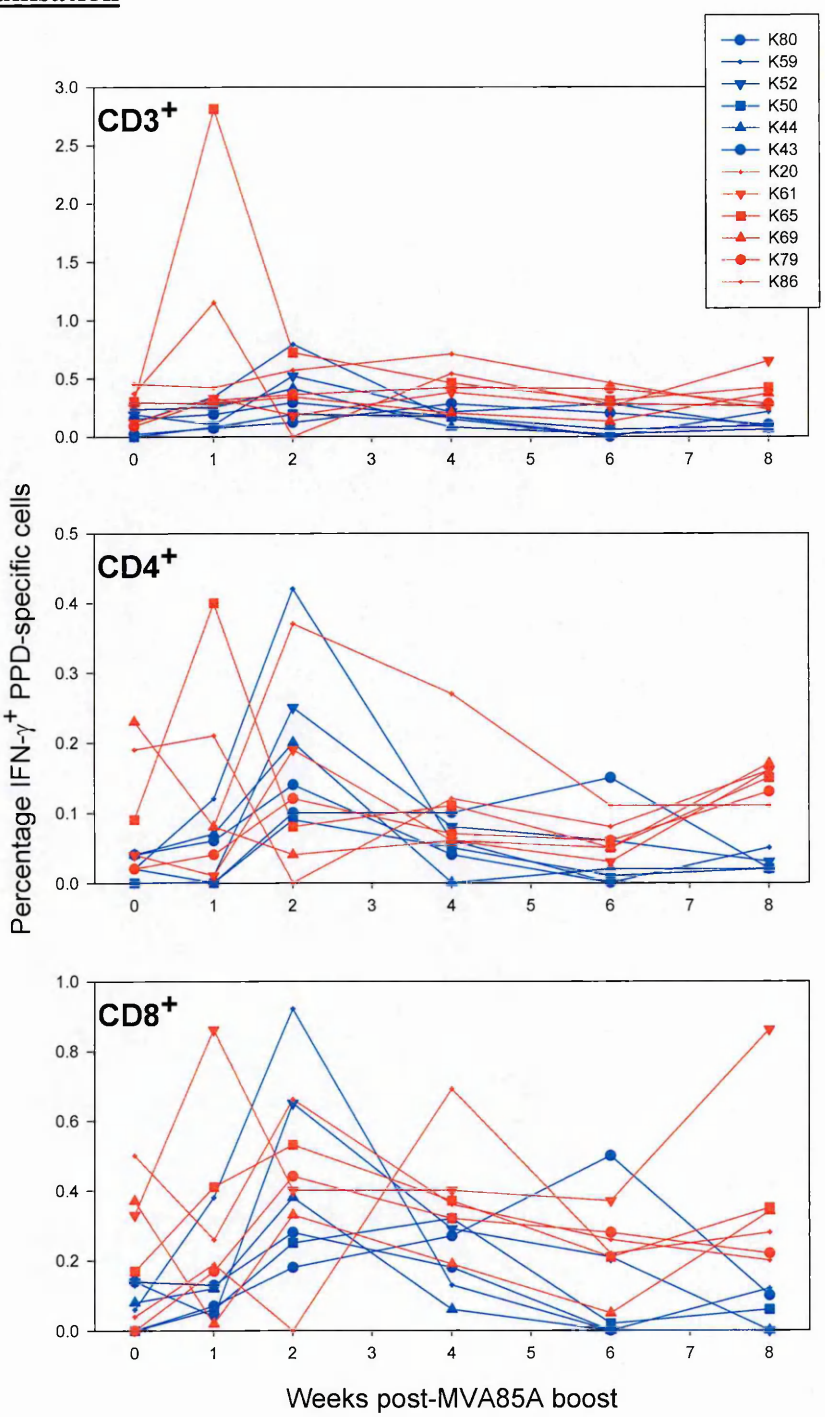
Acknowledgements: Intracellular cytokine results were supplied by Karen Gooch.

8.16 APPENDIX 16: BCG and BCG/MVA85A vaccination and *M. tuberculosis* infection IFN-γ ELISPOT timecourse



IFN-γ ELISPOT timecourse of rhesus macaques after vaccination with BCG and BCG/MVA85A with responses after *M. tuberculosis* infection to PPD (top graph) and summed Ag85A peptides (lower graph).

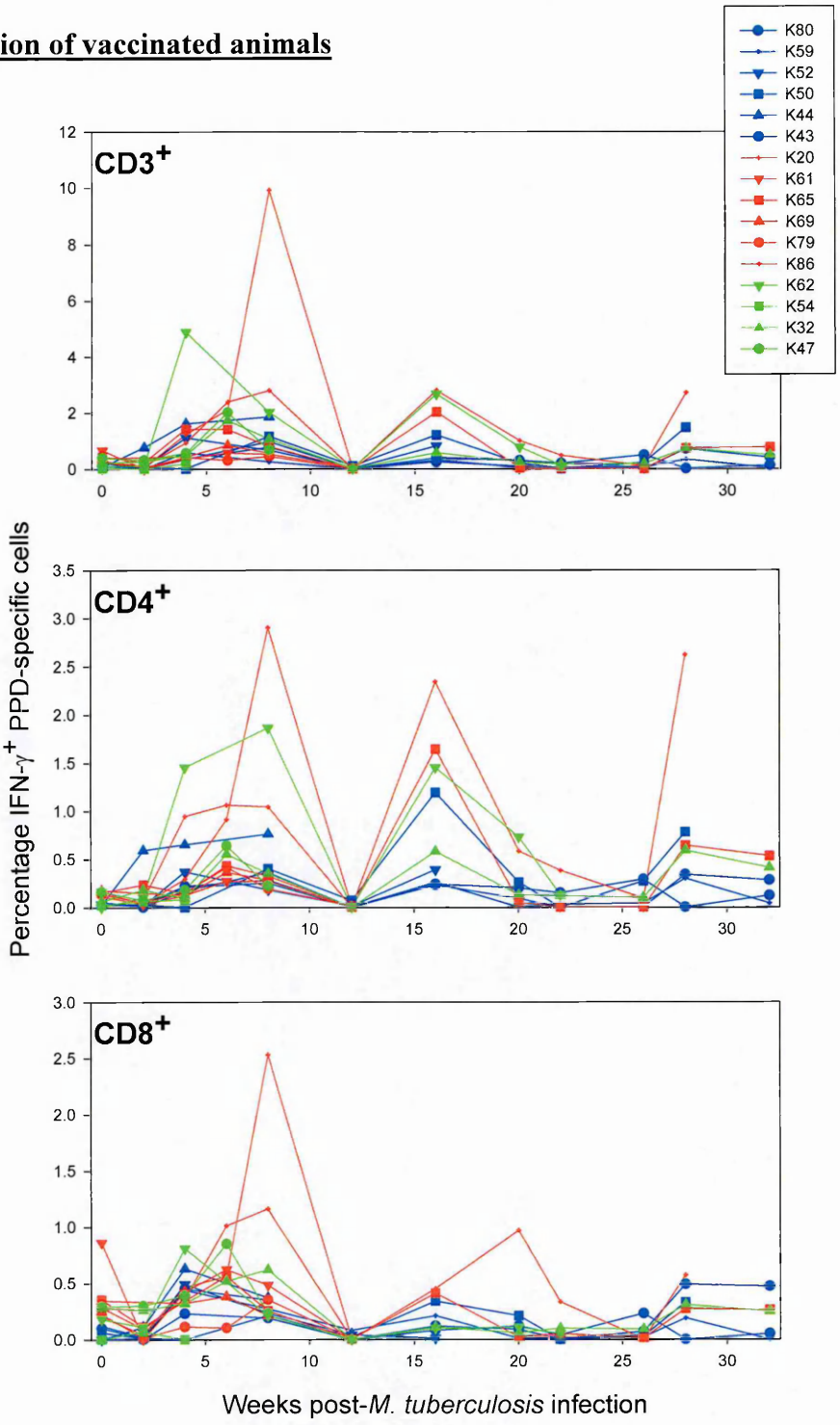
8.17 APPENDIX 17: Intracellular cytokine staining results during MVA85A immunisation



Intracellular cytokine staining results of rhesus macaques that were immunised with MVA85A after previous vaccination with BCG (red lines) or un-MVA85A-immunised (blue lines).

Acknowledgements: Intracellular cytokine results were supplied by Karen Gooch.

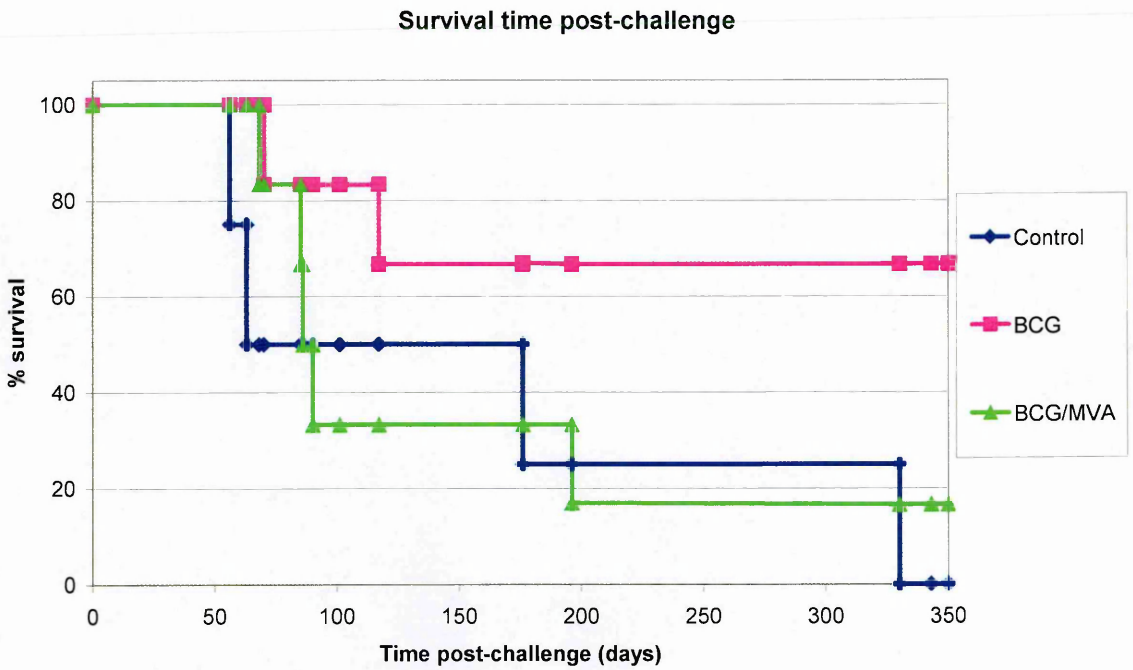
8.18 APPENDIX 18: Intracellular cytokine staining results during *M. tuberculosis* infection of vaccinated animals



Intracellular cytokine staining results of rhesus macaques that were immunised with BCG (blue lines), BCG/MVA85A (red lines) or unvaccinated (green lines) after *M. tuberculosis* infection.

Acknowledgements: Intracellular cytokine results were supplied by Karen Gooch.

8.19 APPENDIX 19: Survival plots of vaccinated rhesus macaques after *M. tuberculosis* challenge



Using log rank Test (non-parametric right sensoring only)

log rank test (p value)

	Control	BCG	BCG MVA
Control	-	-	-
BCG	0.048	-	-
BCG MVA	0.642	0.102	-

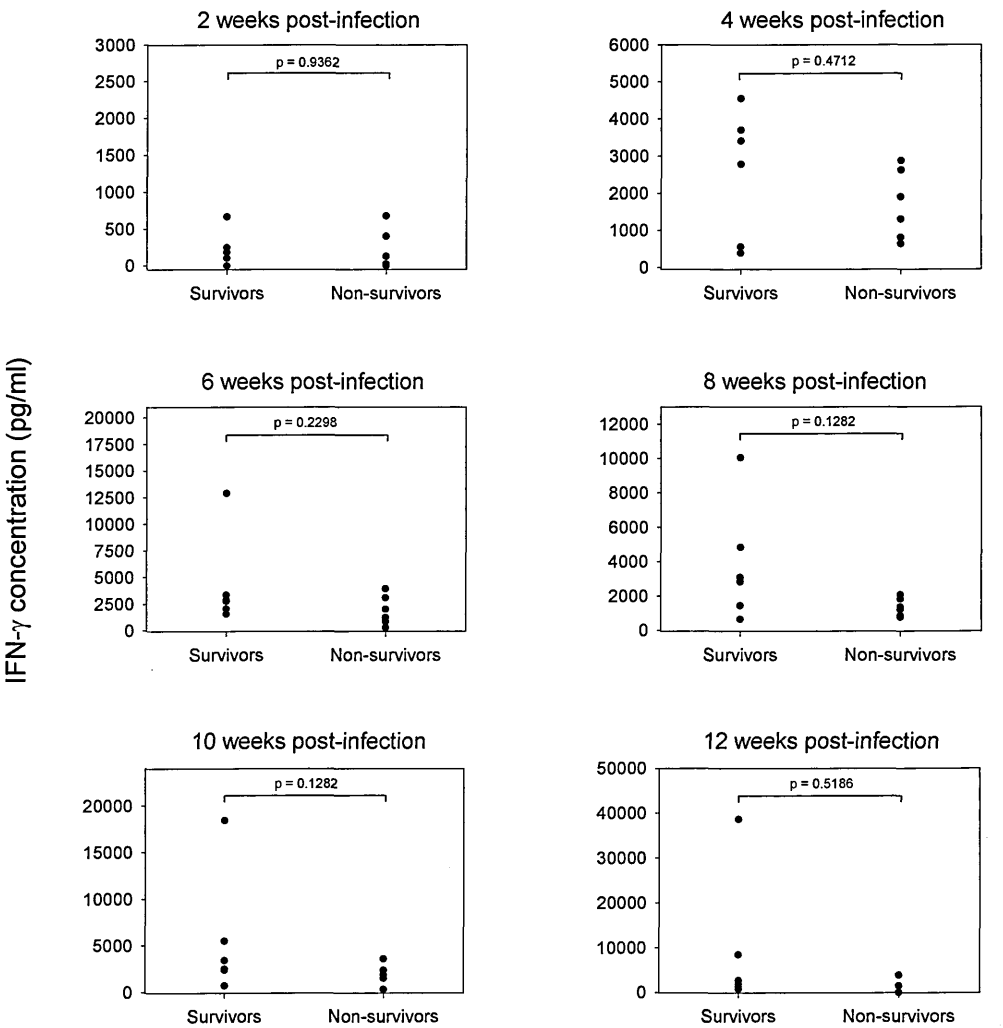
= significantly different from one another
= not significantly different from one another

Survival plot showing percentage survival of animals that were unvaccinated (blue line), BCG vaccinated (pink line) or BCG/MVA85A vaccinated (green line) after aerosol *M. tuberculosis* challenge.

Table shows statistical analysis between groups, with the only significant finding ($P<0.05$) that the BCG-vaccinated group were protected compared to the unvaccinated group.

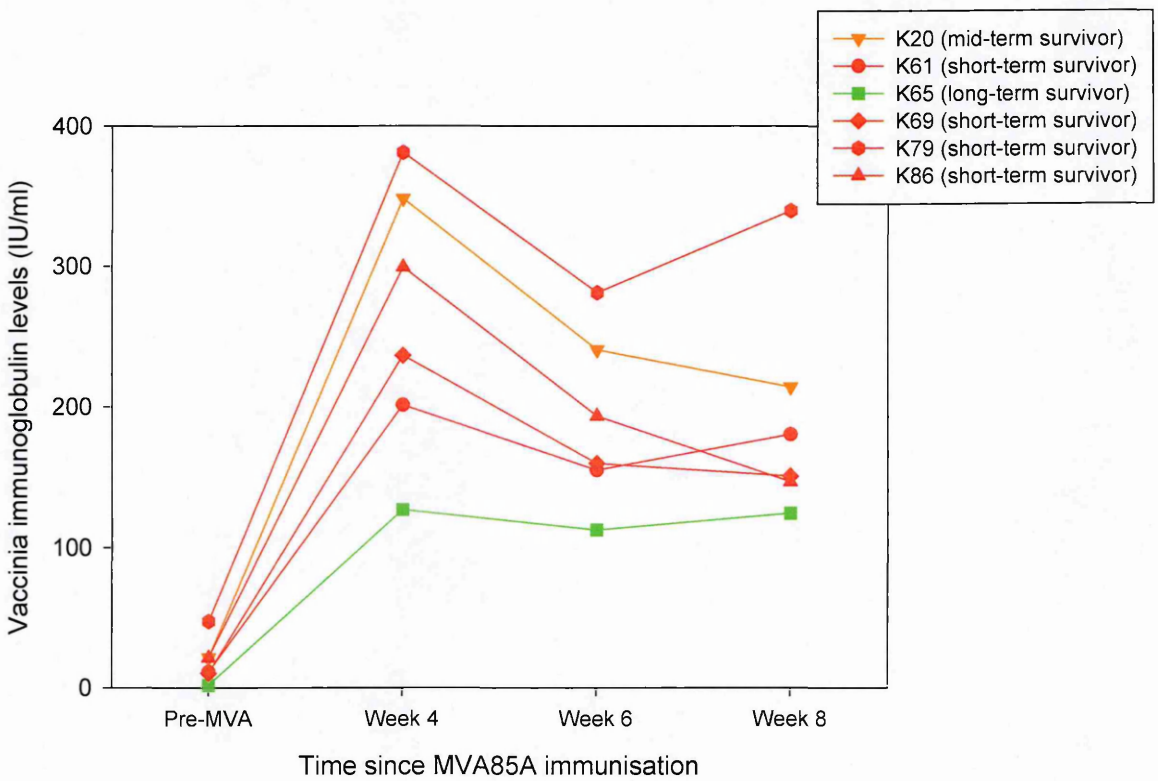
Acknowledgement: Simon Clark assisted in the interpretation of survival data.

8.20 APPENDIX 20: Concentrations of IFN-γ secreted after *M. tuberculosis* challenge of vaccinated rhesus macaques



Concentration of IFN-γ secreted in PPD-stimulated 6 day whole blood supernatants after *M. tuberculosis* challenge of animals vaccinated with BCG and BCG/MVA85A grouped according to survival time. Statistical analysis shows no difference between groups (Mann-Whitney test, significance $P<0.05$).

8.21 APPENDIX 21: Anti-vaccinia antibodies in MVA85A boosted animals compared with survival



Anti-vaccinia antibody responses in MVA85A boosted macaques show that the animal that survived for a year post-challenge had the lowest levels of anti-vector humoral immunity.